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# COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

IN THE MATTER OF: Australian  
Patent Application 696764  
(73941/94). In the name of:  
Human Genome Sciences Inc.  
- and -

IN THE MATTER OF: Opposition  
thereto by Ludwig Institute for  
Cancer Research, under Section  
59 of the Patents Act.

## STATUTORY DECLARATION

I, **Gary Baxter Cox** of Wray and Associates, 239 Adelaide Terrace, Perth WA  
6101, Australia, declare as follows:

1. I am a Registered Patent Attorney, and a member of the firm Wray and Associates, Australian patent attorneys for Human Genome Sciences, Inc., the applicant in this matter.
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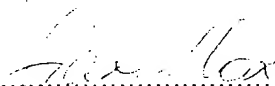
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- 2.23. In this declaration I have only identified those publications that have not already been served by Ludwig Institute for Cancer research in these proceedings. Now produced and shown to me marked "GBC-23 is a Table identifying where all publications in Applicant's evidence in Answer may be found.

AND I make this solemn declaration by virtue of the Statutory Declarations Act, 1959 and subject to the penalties provided by that Act for the making of false statements in statutory declarations, conscientiously believing the statements contained in this declaration to be true in every particular.

DATED this day Thirteenth day of December 2000.

DECLARED at: Perth, Western Australia

BEFORE me: \_\_\_\_\_ )



Gary Baxter Cox

  
\_\_\_\_\_  
Patent Attorney

COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

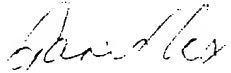
IN THE MATTER OF: Australian  
Patent Application 696764  
(73941/94). In the name of:  
Human Genome Sciences Inc.

- and -

IN THE MATTER OF: Opposition  
thereto by Ludwig Institute for Cancer  
Research, under Section 59 of the  
Patents Act.

Annexure GBC-1

This is **Annexure GBC-1** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.



Gary Baxter Cox

WITNESS:

  
Patent Attorney

PEYTEE KHEO

# Cloning and Characterization of a Novel Human Gene Related to Vascular Endothelial Growth Factor

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This paper describes the cloning and characterization of a new member of the vascular endothelial growth factor (VEGF) gene family, which we have designated VRF for VEGF-related-factor. Sequencing of cDNAs from a human fetal brain library and RT-PCR products from normal and tumor tissue cDNA pools indicate two alternatively spliced messages with open reading frames of 621 and 564 bp, respectively. The predicted proteins differ at their carboxyl ends resulting from a shift in the open reading frame. Both isoforms show strong homology to VEGF at their amino termini, but only the shorter isoform maintains homology to VEGF at its carboxyl terminus and conserves all 16 cysteine residues of VEGF<sub>165</sub>. Similarity comparisons of this isoform revealed overall protein identity of 48% and conservative substitution of 69% with VEGF<sub>189</sub>. VRF is predicted to contain a signal peptide, suggesting that it may be a secreted factor. The VRF gene maps to the DIIS750 locus at chromosome band 11q13, and the protein coding region, spanning ~5 kb, is comprised of 8 exons that range in size from 36 to 431 bp. Exons 6 and 7 are contiguous and the two isoforms of VRF arise through alternate splicing of exon 6. VRF appears to be ubiquitously expressed as two transcripts of 2.0 and 5.5 kb; the level of expression is similar among normal and malignant tissues.

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a secreted, covalently linked homodimeric glycoprotein that specifically activates endothelial tissues (Keck et al. 1989; Leung et al. 1989; Senger et al. 1993). This factor is involved in a variety of physiological processes, including normal angiogenesis, formation of the corpus luteum (Yan et al. 1993), placental development (Sharkey et al. 1993), regulation of vascular permeability (Senger et al. 1993), inflammatory angiogenesis (Sunderkotter et al. 1994), and autotransplantation (Dissen et al. 1994), as well as pathological conditions such as tumor-promoting angiogenesis (Plate et al. 1992; Christofori et al. 1994).

VEGF is a distant relative of the platelet-

derived growth factor (PDGF) gene family with many of the cysteine residues involved in dimerization of these proteins conserved in position (Leung et al. 1989; Keck et al. 1989). A more closely related homolog of VEGF is placenta growth factor (PlGF) (Maglione et al. 1991), which shares 39% amino acid identity and 62% conservative substitution. Furthermore, VEGF and PlGF contain 8 cysteine residues in homologous positions, occur as dimeric proteins, and are therefore likely to have similar tertiary structures (Maglione et al. 1991). VEGF and PlGF have been found to occur together as heterodimers in vivo (DiSalvo et al. 1995). No other closely related homologs of the two proteins have yet been reported.

While attempting to identify candidate genes for multiple endocrine neoplasia type 1 (MEN1), which maps to chromosomal region 11q13 (Lars-

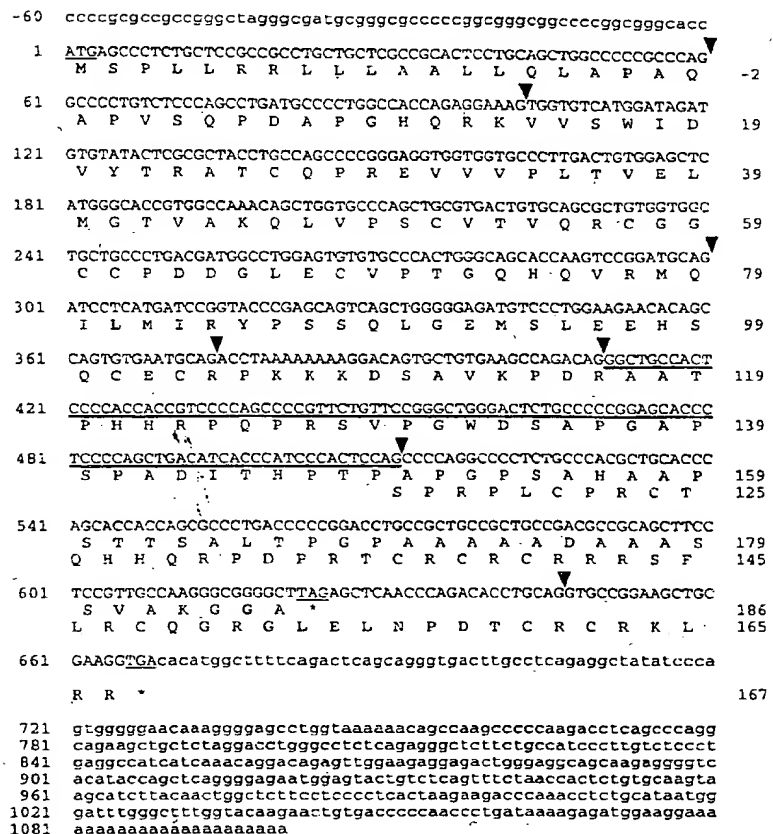
\*Corresponding author.  
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son et al. 1988), we isolated a panel of cDNAs using cosmid cCLGW4 (D11S750) known to map to this region (Larsson et al. 1992). This cosmid was found to contain two previously described genes, *PLCB3* (Weber et al. 1994) and *FKBP2* (Grimmond et al. 1995), as well as novel genes (Lagercrantz et al. 1995a,b). Here we describe the cloning and characterization of a differentially spliced gene from the D11S750 locus, encoding a protein that we have designated VRF (VEGF-related factor), that has striking sequence homology with VEGF.

## RESULTS

### Cloning of VRF cDNAs

The original VRF cDNA, termed pSOM175, was isolated by screening a human fetal brain library (Stratagene) with the cosmid D11S750 (Larsson et al. 1992). cDNA library screening with pSOM175 recovered several partial but overlapping cDNAs for VRF. A composite sequence of the entire coding region was determined and found to consist of a 621-bp open reading frame (ORF), 412 bp of 3' untranslated region (UTR), and 2 bp of 5' UTR (Fig. 1, GenBank accession no. U43368). Attempts to isolate cDNAs with longer 5' UTRs were unsuccessful; therefore, the corresponding region from genomic DNA was sought. An 850-bp *Pst*I restriction fragment from cosmid cCLGW4 (D11S750) (Larsson et al. 1992), which contained exon 1 and an undetermined amount of the 5' UTR, was cloned and partially sequenced, from which -60 bp of the 5' UTR immediately upstream of the initiation codon was determined (GenBank accession no. U43370). To confirm that the sequences upstream of the ATG were 5' UTR, an ExoIII deletion subclone of this region (corresponding approximately to nucleotide positions -250 to -750 with respect to the initiation codon) was used to screen Northern



**Figure 1** Nucleotide and predicted peptide sequences of VRF derived from the cDNA clone pSOM175 (nucleotides -2 to poly(A) tract) or genomic DNA (nucleotides -60 to -3 inclusive). The numbering of nucleotides is given at left starting from the A of the initiation codon. Amino acids are numbered at right, starting from the first residue of the predicted mature protein after the putative signal peptide has been removed. The alternately spliced region is double underlined, and the resulting peptide sequence from each mRNA is included. Start and stop codons are underlined. The positions of intron/exon boundaries are indicated by inverted arrowheads.

blots. Bands corresponding in size to messages obtained with VRF cDNA probes were observed (data not shown).

The putative start codon (Fig. 1) matches the vertebrate consensus sequence [(GCC)GCC(A/G)CCATGG] described by Kozak (1987). Furthermore, based on VRF's homology to VEGF (see below), this ATG is likely to represent the genuine translation start site. However, in-phase stop codons were not identified upstream of this site. An out-of-frame ATG is located at position -37 but is not part of a Kozak consensus sequence.

The 3' end of the cDNA contained a long poly(A) tail that was not preceded by a canonical polyadenylation signal (AATAAA) (Birnstiel et al. 1985); a related sequence, GATAAA, is ~18 nucleotides upstream of the poly(A) tail (Fig. 1).

A second isoform of VRF [designated VRF<sub>167</sub> in keeping with the nomenclature for VEGF (Tischer et al. 1991; Houck et al. 1991) whereby the isoforms are identified by the amino acid lengths of the mature proteins once the signal peptides have been cleaved from the NH<sub>2</sub>-termini] was identified after sequencing PCR products generated from human fetal brain cDNA lysates and RT-PCR products from a renal cell carcinoma. VRF<sub>167</sub> (GenBank accession no. U43369) differs from VRF<sub>186</sub> as a result of a 101-bp deletion between positions +411–+511 (inclusive) in the cDNA encoding the latter (Fig. 1). This not only deletes ~33 amino acids from within VRF<sub>186</sub> but also results in a different carboxy-terminal peptide sequence through the introduction of a frameshift within the ORF that terminates at a new site downstream of the stop codon utilized in generating VRF<sub>186</sub>.

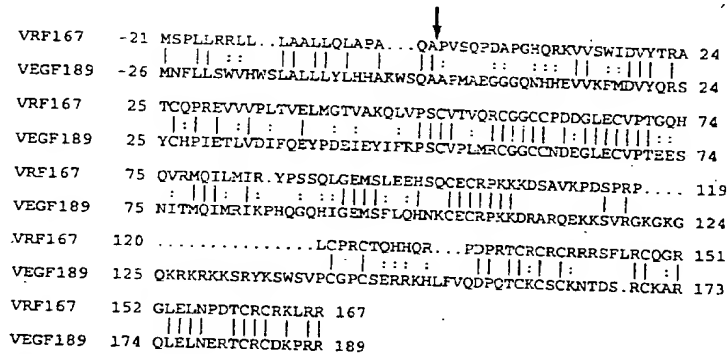
#### Comparison of VRF Isoforms to the VEGF Family

The nucleotide sequences and predicted translation products of VRF cDNAs (Fig. 1) were compared against peptide and nucleotide data bases with BLAST. Four expressed sequenced tags (ESTs) (GenBank accession nos. H28025, H39505, R56770, T08411) were identified as having regions of identity with VRF. Significant homology was observed with VEGF and other gene family members. Nucleotide alignment of the respective cDNAs revealed regions of sequence identity on the order of 59% (124/212 bp). The amino acid homology between VRF<sub>186</sub> and VEGF<sub>189</sub> was 32% identity and 49% similarity over the entire peptide. However, it was notable that no similarity was observed over the carboxy-terminal quarter of the proteins. Sequence alignments of the VRF<sub>167</sub> isoform showed greater overall similarity to members of the VEGF gene family than VRF<sub>186</sub>. Peptide homology comparisons revealed 48% identity and 69% similarity between VEGF and VRF<sub>167</sub>, respectively. This increase in VEGF homology relative to VRF<sub>186</sub> was attributable to additional

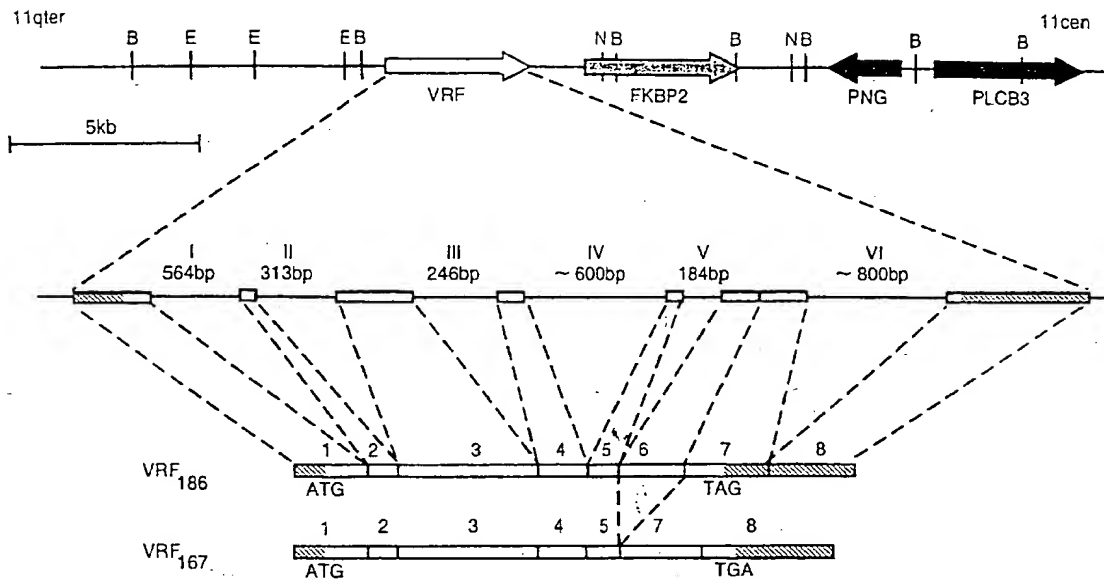
conservation of several distinct regions located toward the carboxyl-terminus of the protein (Fig. 2).

The predicted peptide lengths for the two VRF isoforms (Fig. 1) and the four isoforms of VEGF (Houck et al. 1991) are similar, and a region homologous to the signal peptide at the amino terminus of VEGF (von Heijne 1986; see Fig. 2) is also present in both VRF isoforms. The nomenclature of the VRF isoforms has been derived assuming that the signal peptide is cleaved from the preprotein in the same place as VEGF (Keck et al. 1989; Leung et al. 1989), that is, after alanine 21 in VRF or alanine 26 of VEGF (Fig. 2). Cysteine residues were found to be highly conserved between VRF<sub>167</sub> and other members of the VEGF gene family. Both VRF isoforms contained the 8 cysteines maintained among VEGF, PlGF, and the PDGFs, but an additional 8 cysteine residues were conserved among VRF<sub>167</sub>, VEGF<sub>189</sub>, and PlGF, all of which were located within the divergent carboxy-terminal end of VRF<sub>167</sub>. The striking conservation of number and position of these residues suggests that these three proteins are likely to have very similar tertiary structures.

Several peptide regions within VEGF that are believed to be associated with protein dimerization are maintained between VEGF and both VRF isoforms. The strongest areas of homology include regions located in the mature protein after amino acids 49–71 (PSCVxxxRCGGCCxDxGLECVPT) and 101–107 (CECRPKK) of VRF. In addition, VRF<sub>167</sub> also displays homology to VEGF at the extreme carboxy-terminal end (TCRCxKxRR; amino acids 159–167).



**Figure 2** Homology comparison between VRF<sub>167</sub> and VEGF<sub>189</sub> peptide sequences. The arrow marks the signal peptide cleavage site of VEGF. Identical amino acids are indicated by vertical bars and conservative substitutions by colons. The numbering of amino acids is as described in the legend to Fig. 1.



**Figure 3** Genomic restriction map (B, E, and N represent restriction sites *Bam*HI, *Eco*RI, and *Not*I, respectively) and intron/exon structure of the *VRF* gene together with its orientation relative to other genes within cosmid CCLGW4 (D11S750). Sizes of introns and alternatively spliced RNAs are indicated.

The putative heparin binding clusters located at positions 121–135 of mature VEGF (Leung et al. 1989) are not conserved within the *VRF* isoforms. However, a noncontiguous clustering of basic residues located at the far carboxyl terminus of the VEGF<sub>121</sub> peptide, which is believed to account for its heparin binding ability (Cohen et al. 1995), is present in *VRF*<sub>167</sub>.

#### Characterization of the *VRF* Gene

We have shown above that the *VRF* gene is alter-

nately spliced to yield two major mRNA and protein isoforms. From establishing the intron/exon structure of the protein coding region of this gene (Fig. 3; Table 1) we have found that the *VRF*<sub>167</sub> isoform is generated by the removal of exon 6 from pre-mRNA prior to translation (Fig. 3). The hypothesis that *VRF*<sub>167</sub> (pSOM175-6) was derived by alternate splicing of *VRF* and not another closely related gene was further confirmed by hybridizing a *VRF* cDNA to Southern blots of human genomic DNA. As the genomic region of the *VRF* gene had been restriction mapped previously (Fig. 3), genomic DNA was

digested with restriction enzymes (*Eco*RI, *Bam*HI) that were known not to cut within *VRF*, hybridized with pSOM175-6, and revealed a single band of the expected size. The *VRF* gene was also mapped against a human-hamster hybrid panel, confirming single-gene copy number and localization to 11q13 (data not shown).

The strong conservation of exon/intron organization between members of the VEGF family (Houck et al. 1991; Tis-

**Table 1. Intron/Exon Boundaries of the Human *VRF* Gene**

5'UTR.....	Exon 1 (Xbp*)	GCCCAG	gtacgtgcgg	Intron I	(564bp)
tctcccacag	GCCCT	Exon 2	(43bp)	GGAAAG	gtaataactta
ctgctcccag	TGGTGT	Exon 3	(197bp)	ATGCAG	gtcctgggca
ctgagcacag	ATCCTC	Exon 4	(74bp)	ATGCAG	gtgccagcca
tactttccag	ACCTAA	Exon 5	(36bp)	AGACAG	gtgagtcctt
tctccctag	GGTGC	Exon 6	(101bp)		(No intron)
CCCACTCCAG	CCCCAG	Exon 7	(135bp)	CTGCAG	gtgagcgctc
ccctcctcag	GTGCCG	Exon 8	(431bp)	GGAAGG	

Upper- and lowercase letters denote exonic and intronic sequences, respectively.

\*The 5' end of exon 1 has not yet been determined.

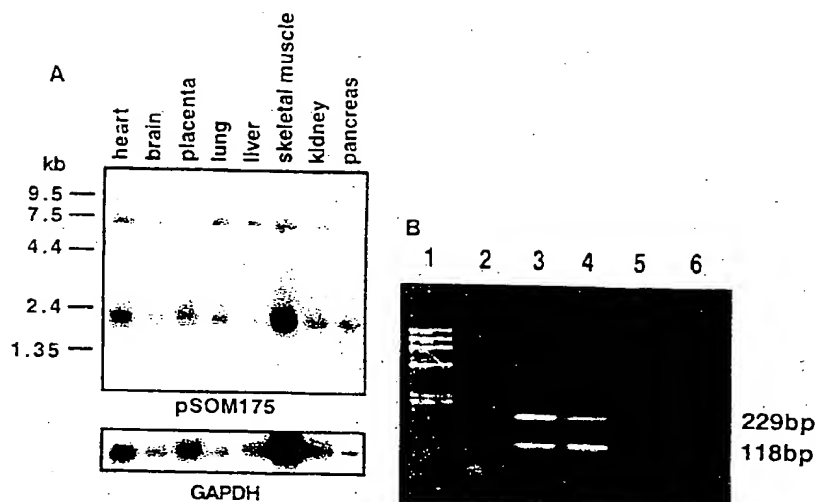
cher et al. 1991) was similarly extended to the genomic structure of *VRF*. In nearly every case, the exon/intron boundaries (Table 1) were found to be in the same location as the *VEGF* gene. The exception was exon 6 of *VRF*, which was contiguous with exon 7 (i.e., no intervening sequence but conservation of the exon/intron boundary position). This suggests that exon 6 in the *VRF* gene is derived from a partially retained intron.

#### Orientation of the *VRF* Gene

The location and orientation of the human *VRF* gene (Fig. 3) within cosmid cCLGW4 (the D11S750 locus), which maps to chromosome 11q13 (Larsson et al. 1992), was determined by PCR between primers from either end of the *VRF* cDNA and a primer located within the 5' end of *FKBP2*. Only an exon 7-specific *VRF* primer and a primer within the 5' UTR of *FKBP2* gave a specific amplification product using both genomic DNA and cCLGW4 as template. Direct sequencing of the termini confirmed the specificity of this product (data not shown).

#### Expression Studies of *VRF*

Northern blot analysis of a total of 20 normal human tissues as well as cultured fibroblasts and lymphoblastoid cell lines revealed that *VRF* was expressed in all samples studied, with no obvious predominance in any tissue after normalization with GAPDH (Fig. 4A). Two bands of 5.5 and 2.0 kb were visible in all samples assayed. We assessed *VRF* expression in normal endocrine tissues, an insulinoma, and a medullary thyroid carcinoma. *VRF* was expressed in all samples, although the level in both tumors was reduced by 50% which corresponded to the loss of one chromosome 11 allele (Weber et al. 1994). Because *VEGF* has been shown previously to be overexpressed in highly malignant tumors (Plate et al. 1992) we assayed levels of *VRF* mRNA in a panel



**Figure 4** (A) Autoradiograph of multiple tissue Northern blot hybridized with the *VRF* cDNA clone pSOM175. Size markers are indicated in kilobases at left. Two transcripts of 5.5 and 2.0 kb were detected in all samples. Results of control hybridization of the same blots using GAPDH cDNA are included in the lower panel. (B) RT-PCR of alternative splice forms of *VRF* in normal human tissue mRNAs. (Lane 1) Size markers ( $\Phi$ X174 DNA cut with *Hae*III); (lane 2) negative control; (lane 3) normal kidney; (lane 4) normal lung; (lane 5) normal pancreas; (lane 6) normal colon.

of 11 glioblastomas, 13 metastasizing and 12 nonmetastasizing breast carcinomas, and 34 renal cell carcinomas. Compared with their normal counterparts or nonmalignant cell lines, elevated transcription was not found in any of the tumors.

As it was not possible to differentiate between the alternately spliced *VRF*<sub>186</sub> and *VRF*<sub>167</sub> mRNAs by Northern analysis owing to the small size difference (101 nucleotides), RT-PCR was performed to confirm further the presence of both messages in normal and tumor tissues. A region corresponding to the carboxy-terminal end of the ORF (nucleotide positions +362–+590; see Fig. 1) was amplified from a panel of matched human normal tissue/tumor mRNAs with two major products being identified (Fig. 4B). Direct sequencing of these products confirmed that they represented the two different *VRF* isoforms.

#### DISCUSSION

We have cloned and characterized a new member of the *VEGF* gene family, which we have designated *VRF*. The strong homology between *VEGF*, *PlGF*, and *VRF* reflects conservation of structural



motifs important for peptide function (i.e., homo/heterodimerization and heparin binding).

As yet, the various roles of VRF *in vivo* remain to be elucidated. We have shown here that VRF possesses strong homology to several angiogenic factors, and investigations into its effect on endothelial cell function are ongoing. Furthermore, its ubiquitous expression pattern suggests that its role may extend beyond the endothelium. In light of the recent report that VEGF and PlGF form heterodimers *in vivo* (DiSalvo et al. 1995), it is possible that VRF may also interact with one or both of these factors in a similar fashion. As VRF proteins have divergent carboxy-terminal ends, with the longer isoform lacking some of the motifs involved in VEGF stability and function, it is tempting to speculate that this isoform could act as an antagonist/regulator of the shorter isoform.

Recent studies of VEGF function have reported the importance of heparin binding that is involved in dimerization and transport and assists in binding of the protein to some receptors such as *flt1* (Gengrinovitch et al. 1995). One of the major heparin binding domains (basic cluster of residues at position 121–135; see Fig. 2) of VEGF (Leung et al. 1989; Ferrara et al. 1992) is absent from both VRF isoforms. However, VRF<sub>167</sub> may still be capable of heparin binding through a region of basic amino acids at its carboxyl terminus, provided the tertiary structure of the protein allows the clustering of these noncontiguous residues.

The strong sequence homology between VEGF, PlGF, and VRF reflect conservation of genomic structure between their genes with a similar number of exons, near identical intron/exon borders, and the existence of alternately spliced mRNA, particularly involving exon 6. One significant difference between VRF and the other VEGF gene family members is that the alternately spliced messages of VRF reported here give rise to proteins with different carboxyl termini. We show that this phenomenon arises through the retention or deletion of exon 6. Retention of intervening sequences in mRNA has been documented as a post-translational regulatory mechanism in several genes including P-transposase in *Drosophila* (for review, see Maniatis 1991) and bovine growth hormone pre-mRNA (Dirksen et al. 1995). The retention of an intron that results in a frameshift and different carboxyl termini is an uncommon phenomenon but has been reported recently for the  $\beta$ 1-adrenergic receptor in the tur-

key (Wang and Ross 1995). In the case of  $\beta$ -adrenergic receptor, intronic retention gives rise to two receptor types and is involved in providing tissue specificity. The mechanisms that control intron retention in pre-mRNAs have been studied for some genes and involve specific splicing repressor factors (for review, see Maniatis 1991). Thus, studies to determine the possible role of such factors in regulation of the VRF gene appear warranted.

While the elucidation of all the possible roles of VRF continues, it is tempting to speculate that the two VRF protein isoforms act in an antagonistic or self-regulatory manner, similar to that reported for the turkey  $\beta$ -adrenergic receptor isoforms (Wang and Ross 1995).

The genomic localization of VRF at D11S750 places it within a 900-kb region known to contain the *MEN1* gene (Weber et al. 1994). In a large panel of tumors of endocrine and nonendocrine origin, a reduction in expression of VRF was only observed in those endocrine tumors known to be hemizygous for chromosome 11q, suggesting this was a gene dosage effect. Although VRF has not yet been excluded as a *MEN1* candidate by mutation analysis, its putative role as a growth factor makes it an unlikely candidate for the *MEN1* tumor suppressor gene.

## METHODS

### cDNA Cloning Sequencing, and Analysis

Screening of a human fetal brain library (Stratagene) with the cosmid D11S750 (Larsson et al. 1992) was performed as described (Viskochil et al. 1992). The 1.1-kb insert of SOM175 was used as a probe to isolate other cDNAs from a human fetal spleen library (Stratagene). The isolated cDNAs were sequenced on both strands using standard manual sequencing and automated sequencing protocols (PRISM, Applied Biosystems, Inc., model 373A). Oligonucleotides, nested deletions (Erase-a-base, Promega), and specific cDNA subclones were generated to complete total cDNA sequences. PCR products generated from the cDNAs were first purified from agarose gels (Qiagex gel purification columns, Qiagen) and then sequenced. Sequences were compared with the current GenBank data base at the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (Altschul et al. 1990). Peptide homology alignments were performed using the program BESTFIT (GCG Wisconsin).

### Northern Blot Analysis

Multiple tissue Northern blots (Clontech) containing poly(A)<sup>+</sup> RNA from heart, brain, placenta, lung, liver, skel-

etal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes were used to determine expression of *VRF* in normal human tissues. Northern filters from renal cell carcinomas and breast carcinomas were kindly provided from Drs. Ulf Bergerheim, Moraima Zelada, and Esther Schmidt. The extraction of poly(A)<sup>+</sup> RNA from normal adrenal, pancreas, thyroid, parathyroid, kidney, fibroblasts, lymphoblastoid cell lines, and endocrine tumors, the preparation of blots, and the hybridization conditions with cDNA probes were performed as described (Weber et al. 1994).

## RT-PCR

Total RNA was isolated from a panel of human tumors and matching normal tissues (colon, lung, liver, kidney, pancreas), and cDNA synthesis reactions were carried out using 5 µg of RNA, random hexamers, and AMV reverse transcriptase (Promega) following methods recommended by the manufacturer. Five hundred nanograms of reverse-transcribed cDNA mixture (1 µl) was used in a PCR reaction to detect possible alternately spliced messages. Alternatively, 1 µl of high titer (>10<sup>9</sup> PFU/ml) cDNA library lysate was used as a template. The primers were 362F (5'-AGTGTGAATGCAGACCT-3') and 590R (5'-GCCGCGGCAGCGGCAGCGG-3'). PCR products were visualized after electrophoresis through high percentage (3%) agarose gels stained with ethidium bromide. Alternately spliced products were confirmed by direct sequencing as described above.

## Genomic Sequencing and Intron/Exon Mapping of the *VRF* Gene

Cosmid cCLGW4 (Larsson et al. 1992) was used as template and sequenced on both strands using both manual dideoxy sequencing methods and automated fluorescently labeled "dye terminator" (PRISM, Applied Biosystems, Inc.) cycle sequencing as described above, except that 2 µg of cosmid template and 20 pmol of primer were used in each reaction. PCR products from genomic DNA were also sequenced using dye-terminator cycle sequencing after purification of products from agarose gels using Qiagex gel purification columns (Qiagen). An oligonucleotide (19F, 5'-CGCCTGCTGCTCGCCGCACT-3') was made to a region corresponding to nucleotides 19–38 with respect to the initiation codon, end-labeled with [ $\gamma$ -<sup>32</sup>P] dATP, and hybridized to a Southern blot of a series of shotgun-cloned *Pst*I restriction fragments from cosmid cCLGW4 subcloned into pBluescript KS- (Stratagene). A single hybridizing clone with an 850 bp insert was sequenced on both strands as described above.

Intervening sequences were located by sequencing of cosmid cCLGW4 (Larsson et al. 1992) using oligonucleotide primers from the *VRF* cDNA sequence determined above. Comparison of cDNA and cosmid sequences revealed the exact location of each exon/intron boundary. The size of each intron was then determined by PCR amplification using flanking exonic primers and cCLGW4 or genomic DNA as template. Amplified products were gel purified and directly sequenced to confirm intron/exon boundaries. The intron sizes were determined either by

complete sequencing of the intervening sequence or estimated by electrophoresis through high percentage agarose gels.

## ACKNOWLEDGMENTS

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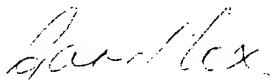
**IN THE MATTER OF:** Australian  
Patent Application 696764  
(73941/94). In the name of:  
Human Genome Sciences Inc.

- and -

**IN THE MATTER OF:** Opposition  
thereto by Ludwig Institute for Cancer  
Research, under Section 59 of the  
Patents Act.

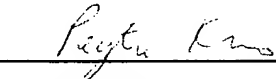
**Annexure GBC-2**

This is **Annexure GBC-2** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.



Gary Baxter Cox

WITNESS:



Patent Attorney

PEYTEE KHOO

## Characterization of the Murine VEGF-Related Factor Gene

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We describe here the molecular cloning and characterization of the murine homolog of the human vascular endothelial growth factor-related factor (VRF) gene. cDNAs for two alternatively spliced forms of the murine *vrf* gene have been isolated, the putative translation products of which differ at their carboxyl termini due to a shift in reading frame caused by insertion, or lack thereof, of exon 6, in a similar fashion to human VRF (hVRF). The message lacking exon 6 encodes a protein (mvrf<sub>167</sub>) with 86% identity and 92% conservation of amino acid residues with hVRF. The protein coding region of the gene spans approximately 5kb of genomic DNA and is composed of 8 exons ranging in size from 36 to 398bp. The genomic structure of murine *vrf* is highly conserved with the human homolog in relation to position of splice junctions and the presence of contiguous exons 6 and 7. A short polymorphic AC repeat is present in the 3' untranslated region of murine *vrf*. A major band of approximately 1.3kb was expressed in all adult mouse tissues examined. © 1996 Academic Press, Inc.

Angiogenesis is an important physiological process in embryonic development, somatic growth, wound healing, tissue and organ regeneration and cyclical growth of the corpus luteum and endometrium [reviewed in 1, 2]. The growth and differentiation of capillary vessels is a complex process in which endothelial cells migrate, proliferate and are involved in degradation of the extracellular matrix and tube formation. The formation of capillaries is also associated with a number of pathological conditions which include tumor growth [3-5], diabetes-related retinopathy [6], atherosclerosis and rheumatoid arthritis [7]. Vascular endothelial growth factor (VEGF), is a mitogen that is highly selective for endothelial cells [8, 9], and belongs to a family of growth factors that includes platelet derived growth factor (PDGF) -A and PDGF-B [8, 9], and placenta growth factor (PIGF) [10]. Native VEGF is normally found as a homodimer and is one of the ligands for the *flt/Flk* family of receptor tyrosine kinases found on the surface of vascular endothelial cells [reviewed in 11, 12]. Heterodimers of VEGF and PIGF were identified recently *in vivo* and found to be mitogenic [13].

The VEGF transcript is differentially spliced to produce four distinct peptides that have variable biological properties and activities [reviewed in 14, 15]. We recently cloned and characterized a gene from humans that encodes a VEGF-related factor (VRF) [16]. Human VRF (hVRF) transcripts are alternately spliced with two major isoforms (hVRF<sub>186</sub> and hVRF<sub>167</sub>) being present. The smaller isoform (hVRF<sub>167</sub>) lacks an 101bp exon (exon 6) and maintains strong amino acid sequence homology to VEGF throughout the peptide while the larger message possesses a divergent alanine-rich carboxyl terminus. In this report we describe the isolation of the homologous gene from mouse

Sequences presented in this article have been submitted to the GENBANK database and appear under accession numbers U43836 and U43837.

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**Abbreviations:** hVRF - human VEGF-related factor; mvrf - murine VEGF-related factor; PDGF - platelet derived growth factor; PIGF - placenta growth factor; UTR - untranslated region; VEGF - vascular endothelial growth factor; VRF - human VEGF-related factor gene; *vrf* - murine VEGF-related factor gene.

which likewise encodes two m  
exon 6.

**Isolation of cDNAs.** Murine *vrf* clones  
Primary phage from high density filters  
generated by PCR from an human VRF c  
of nylon membranes (Hybond-N) were c  
plaques were picked, purified and excise

**Isolation of genomic clones.** Genomic  
II vector (Stratagene). High density filter  
PCR amplification of the nucleotide 233  
re-screened with filters containing 400-  
kit or by ZnCl<sub>2</sub> purification [18].

**Nucleotide sequencing and analysis.** c  
primers with Applied Biosystems Inc.  
specifications. Sequences were analyzed  
were performed using the program BE5

**Identification of intron/exon bound**  
PCR with mouse genomic DNA or mu  
introns were derived from the human  
annealing temperatures 5-10°C below  
phoresis and gel purified using QIAquick  
from these products. In addition, some  
exon boundaries were identified by con

**Northern analysis.** Total cellular RN  
liver, muscle) using the method of Chom  
a nylon membrane (Hybond N, Amersh  
0.1 × SSC (20 × SSC is 3M NaCl/0.3M  
at -70°C for 1-3 days.

### Characterization of Murine *vrf*

Murine *vrf* homologs were i  
cDNA clone. Five clones of si  
cDNA sequences were compiled  
open reading frame (621bp or  
(379bp), as well as 189bp of th

The predicted initiation codon  
other ATG codons (positions -2  
upstream and out of frame with

The predicted N-terminal sig  
identity (17/21 amino acids). P  
(Fig. 2). These data suggest that  
as a growth factor.

As with hVRF, two open rea  
screening. Four of five clones  
number U43837) and lacked a  
predicted peptide sequences of  
corresponding human isoforms.

The message encoding mvrf  
position +622, towards the end  
terminates downstream of the +1

which likewise encodes two major protein isoforms which arise through alternative splicing of exon 6.

## MATERIALS AND METHODS

**Isolation of cDNAs.** Murine *vrf* clones were selected from a lambda Zap new born whole brain cDNA library (Stratagene). Primary phage from high density filters ( $5 \times 10^4$  pfu/plate) were identified by hybridization with a 682bp  $^{32}$ P-labelled probe generated by PCR from an human *VRF* cDNA (pSOM175) as described previously [16]. Hybridization and stringent washes of nylon membranes (Hybond-N) were carried out at 65°C under conditions described by Church and Gilbert [17]. Positive plaques were picked, purified and excised *in vivo* to produce bacterial colonies containing cDNA clones in pBluescript SK-.

**Isolation of genomic clones.** Genomic clones were isolated from a mouse strain SV129 library cloned in the lambda Fix II vector (Stratagene). High density filters ( $5 \times 10^4$  pfu/filter) were screened with a 563bp  $^{32}$ P-labelled probe generated by PCR amplification of the nucleotide 233-798 region of the murine *vrf* cDNA (see Fig. 1). Positive clones were plugged and re-screened with filters containing 400-800 pfu. Large scale phage preparations were prepared using the QIAGEN lambda kit or by  $ZnCl_2$  purification [18].

**Nucleotide sequencing and analysis.** cDNAs were sequenced on both strands using a variety of vector-based and internal primers with Applied Biosystems Incorporated (ABI) dye terminator sequencing kits according to the manufacturer's specifications. Sequences were analyzed on an ABI Model 373A automated DNA sequencer. Peptide homology alignments were performed using the program BESTFIT (GCG, Wisconsin).

**Identification of intron/exon boundaries.** Identification of exon boundaries and flanking regions was carried out using PCR with mouse genomic DNA or murine *vrf* genomic lambda clones as templates. The primers used in PCR to identify introns were derived from the human *VRF* sequence [16] and to allow for potential human-mouse sequence mismatches annealing temperatures 5-10°C below the estimated  $T_m$  were used. All PCR products were sized by agarose gel electrophoresis and gel purified using QIAquick spin columns (Qiagen) and the intron/exon boundaries were sequenced directly from these products. In addition, some splice junctions were sequenced from subcloned genomic fragments of *vrf*. Intron/exon boundaries were identified by comparing cDNA and genomic DNA sequences.

**Northern analysis.** Total cellular RNA was prepared from a panel of fresh normal adult mouse tissues (brain, kidney, liver, muscle) using the method of Chomezynski and Sacchi [19]. 20µg of total RNA were electrophoresed, transferred to a nylon membrane (Hybond N, Amersham) and hybridised under standard conditions [17]. Filters were washed at 65°C in  $0.1 \times$  SSC ( $20 \times$  SSC is 3M NaCl/0.3M trisodium citrate), 0.1% SDS and exposed to X-ray film with intensifying screens at -70°C for 1-3 days.

## RESULTS AND DISCUSSION

### Characterization of Murine *vrf* cDNAs

Murine *vrf* homologs were isolated by screening a murine cDNA library with a human *VRF* cDNA clone. Five clones of sizes varying from 0.8-1.5kb were recovered and sequenced. The cDNA sequences were compiled to give a full length 1233bp cDNA sequence covering the entire open reading frame (621bp or 564bp depending on the splice form, see below) and 3' UTR (379bp), as well as 189bp of the 5' UTR (Fig. 1, GENBANK accession number U43836).

The predicted initiation codon matched the position of the start codon in human *VRF* [16]. Two other ATG codons (positions -34 and -80) and a termination codon (position -41) were observed upstream and out of frame with the putative initiation codon.

The predicted N-terminal signal peptide of hVRF [16] appears to be present in mvrf with 81% identity (17/21 amino acids). Peptide cleavage within mvrf is expected to occur after residue 21 (Fig. 2). These data suggest that mature mvrf is secreted and could therefore conceivably function as a growth factor.

As with hVRF, two open reading frames (ORFs) were detected in cDNAs isolated by library screening. Four of five clones were found to be alternatively spliced (GENBANK accession number U43837) and lacked an 101bp fragment homologous to exon 6 of hVRF [16]. The predicted peptide sequences of the two isoforms of mvrf were determined and aligned with the corresponding human isoforms (Fig. 2).

The message encoding mvrf<sub>136</sub> contains a 621bp ORF with coding sequences terminating at position +622, towards the end of exon 7 (Fig. 1). The smaller message encoding mvrf<sub>167</sub> actually terminates downstream of the +622 TAG site due to a frame shift resulting from splicing out of the

### ted Factor Gene

d.\* Ginters Silins,\*  
olas Hayward\*<sup>1</sup>

am, Queensland Institute of Medical  
ne, Clinical Genetics Unit, Karolinska

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at their carboxyl termini due to a  
ar fashion to human VRF (hVRF).  
92% conservation of amino acid  
tely 5kb of genomic DNA and is  
of murine *vrf* is highly conserved  
sence of contiguous exons 6 and  
of murine *vrf*. A major band of  
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development, somatic growth,  
th of the corpus luteum and  
capillary vessels is a complex  
involved in degradation of the  
aries is also associated with a  
5], diabetes-related retinopathy  
ial growth factor (VEGF), is a  
belongs to a family of growth  
PDGF-B [8, 9], and placenta  
homodimer and is one of the  
the surface of vascular endo-  
were identified recently *in vivo*

inct peptides that have variable  
tly cloned and characterized a  
5]. Human VRF (hVRF) tran-  
d hVRF<sub>167</sub> being present. The  
ins strong amino acid sequence  
possesses a divergent alanine-  
homologous gene from mouse

e and appear under accession numbers

search, P.O. Royal Brisbane Hospital.

ated factor; PDGF - platelet derived  
ascular endothelial growth factor; VRF

-189 ctcaggccgcgtcgtctgcgcgcgttgcgtctgcgcgcagggcctcgggagggggcc  
 -129 gccgaggagccgccccctgcgccccgccccgggtccccgggtccgcgccatggggtctgt  
 -69 gctgcgcgcgcgcgcgcacgcgcgcgggttagggccatgcgggcgcctccgcgcctgcgcc  
 -9 cgcggcaccATGAGCCCCCTGCTCCGTCGCCTGCTGCTTGTGCACTGCTGCAGCTGGCT  
                   M S P L L R R L L L V A L L Q L A -5  
 52 CGCACCAGGCCCTTGTGTCCAGTTTGATGGCCCCAGCCACCAGAAGAAAGTGGTGCCA  
   R T Q A P V S Q F D G P S H Q K K V V P 16  
 112 TGGATAGACGTTTATGCACGTGCCACATGCCAGCCCAGGGAGGTGGTGGTGCCTCTGAGC  
   W I D V Y A R A T C Q P R E V V V P L S 36  
 172 ATGGAATCATGGGCAATGTGGTCAAACAACCTAGTGCCAGCTGTGTGACTGTGCAGCGC  
   M E L M G N V V K Q L V P S C V T V Q R 56  
 232 TGTGGTGGCTGCTGCCCTGACGATGGCCTGGAATGTGTGCCCACTGGGCAACACCAAGTC  
   C G G C C C P D D G L E C V P T G Q H Q V 76  
 292 CGAATGCAGATCCTCATGATCCAGTACCCGAGCAGTCAGCTGGGGGAGATGTCCCTGGAA  
   R M Q I L M I Q Y P S S Q L G E M S L E 96  
 352 GAACACAGCCAATGTGAATGCAGACCAAAAAAAAAAGGAGAGTGTGTGAAGCCAGACAGG  
   E H S Q C E C R P K K K E S A V K P D R 116  
 412 GTTGCCATACCCACCAACCGTCCCCAGCCCCGCTCTGTTCCGGGCTGGGACTCTACCCCG  
   V A I P H H R P Q P R S V P G W D S T P 136  
 472 GGAGCATCCTCCCCAGCTGACATCATCCATCCCCTCCAGCCCCAGGATCCTCTGCCCGC  
   G A S S P A D I I H P T P A P G S S A R  
                                   S P R I L C P 156  
 532 CTTGCACCCAGCGCGCTCAACGCCCTGACCCCGGACCTGCCGCTGCCGCTGCAGACGCC  
   L A P S A V N A L T P G P A A A A A D A 175  
   P C T Q R R Q R P D P R T C R C R C R R 142  
 592 GCCGTTCTCTCCATTGCCAAGGGCGGGGCTTAGAGCTCAACCAGACACCTGTAGGTGCC  
   A A S S I A K G G A \* 186  
   R R F L H C Q G R G L E L N P D T C R C 162  
 652 GGAAGCCGCGAAAGTGacaagctgctttccagactccacgggccccgggtgcttttatggc  
   R K P R K \* 167  
 712 cctgcttcacagggagaagagtggagcacaggcgaaacctcctcagtcctgggaggtcactg  
 772 cccaggacctggaccttttagagagctctctcgccatcttttatctcccagagctgcc  
 832 tctaacaattgtcaaggaaacctcatgtctcacctcaggggccagggtactctctactta  
 892 accacccttggtcaagtgcagcatcttctggctgggtgctctccctcactatgaaaaccca  
 952 aactctactaataacgggatttgggtctgttatgataactgtgacacacacacacac  
 1012 cactctctgataaaagagatggaagacactaacacaaaaaaaaaaaaaaaaaaaaaaaaa

**FIG. 1.** Nucleotide and predicted peptide sequences derived from murine *vrf* cDNA clones. Numbering of nucleotides is given on the left, starting from the A of the initiation codon. Amino acids are numbered on the right, starting from the first residue of the predicted mature protein after the putative signal peptide has been removed. The alternately spliced region is double underlined and the resulting peptide sequence from each mRNA is included. A potential polyadenylation signal is indicated in boldface. Start and stop codons of *mvrf*<sub>167</sub> and *mvrf*<sub>186</sub> are underlined and a polymorphic AC repeat in the 3' UTR is indicated by a stippled box. The positions of intron/exon boundaries are indicated by arrowheads.

101bp exon 6 and the introduction of a stop codon (TGA) at position +666, near the beginning of exon 8 (Fig. 1).

The mvrf<sub>186</sub> protein has strong homology to the amino and central portions of VEGF while the carboxyl end is completely divergent and is alanine rich. The mvrf<sub>167</sub> possesses these similarities

## A

hVRF167	-21	MSPLLRLR	
mvrf167	-21	MSPLLRLR	
hVRF167	30	EVVVPLTV	
mvrf167	30	EVVVPLSM	
hVRF167	80	ILMIRYPS	
mvrf167	80	ILMIQYPS	
hVRF167	130	RPDPRTCF	
mvrf167	130	RPDPRTCF	

## B

```

hVRF186      116  RAATPHHF
              |||||
mvrf186      116  RVAIPHHF

hVRF186      166  TPGPAAAF
              |||||
mvrf186      166  TPGPAAAF

```

FIG. 2. BESTFIT alignments of human hVRF<sub>180</sub> from the point where the sequences are marked with vertical bars and conserved site of human and murine VRF.

```

mvrfl67      -21  MSPLLRL
                |
mvegfl188    -26  MNFLLSL
                |
mvrfl67      25   TCQPRE
                | |
mvegfl188    24   YCRPIE
                | |
mvrfl67      75   QVRMOI
                : | |
mvegfl188    74   NITMQI
                | |
mvrfl67     119   .....
mvegfl188    124  QKRKRK
                |
mvrfl67     152  GLELNP
                | |
mvegfl188    173  GLELNE
                | |

```

**FIG. 3.** BESTFIT alignment of my cleavage site of mvegfl. Identical amino acids are boxed. Numbering of amino acids is as described in Fig. 1.

cagggtcgggagggggcc  
 cccgcgcatggggctctg  
 cgtcccgccctcgccc  
 GCACTGCTGCAGCTGGCT  
 A L L Q L A -5  
 CAGAAGAAAGTGGTGCCA  
 Q K K V V P 16  
 GGTGGTGGTGCCTCTGAGC  
 V V V P L S 36  
 TGTGTGACTGTGCAGCGC  
 C V T V Q R 56  
 ACTGGGCAACACCAAGTC  
 T G Q H Q V 76  
 GGGGAGATGTCCCTGGAA  
 G E M S L E 96  
 GCTGTGAAGCCAGACAGG  
 A V K P D R 116  
 GGCTGGGACTCTACCCCG  
 G W D S T P 136  
 CCAGGATCCTCTGCCCGC  
 P G S S A R 156  
 P R I L C P 122  
 GCTGCCGTCGACAGCGC  
 A A A A D A 176  
 C R C R R 142  
 GAGACACCTGTAGGTGCC  
 186  
 P D T C R C 162  
 cccggtgcttttatggc  
 167

cna clones. Numbering of nucleotides  
 numbered on the right, starting from the  
 been removed. The alternately spliced  
 included. A potential polyadenylation  
 declined and a polymorphic AC repeat  
 ies are indicated by arrowheads.

on +666, near the beginning of

al portions of VEGF while the  
 167 possesses these similarities

## A

hVRF167 -21 MSPLLRLLLAALLQLAPAPVVSQPDAPGHQKVVSWIDVYTRATCQPR 29  
 mvr167 -21 MSPLLRLLLVALLQLARTQAPVSQFDGPGSHQKVVFWIDVYARATCQPR 29  
 hVRF167 30 EVVVPLTVELMGTVAKQLVPSCVTVQRCGGCCPDDGLECVPTGQHVRMQ 79  
 mvr167 30 EVVVPLSMELMGNVVKQLVPSCVTVQRCGGCCPDDGLECVPTGQHVRMQ 79  
 hVRF167 80 ILMIRYPSSQLGEMSL E E H S Q C E C R P K K K D S A V K P D S P R P L C P R C T Q H H Q 129  
 mvr167 80 ILMIQYPSSQLGEMSL E E H S Q C E C R P K K K E S A V K P D S P R I L C P P C T Q R R Q 129  
 hVRF167 130 RPDPRTCRCRCRRRSLRCQGRGLELNPDTCRCKRLRR\* 167  
 mvr167 130 RPDPRTCRCRCRRRFLHCQGRGLELNPDTCRCKPRK\* 167

## B

hVRF186 116 RAATPHHRPQPRSVPGWDSAPGAPSPADITHPTAPGSAHAAPSTTSAL 165  
 mvr186 116 RVAIPHHRPQPRSVPGWDSTPGASSPADIIHPTAPGSSARLAPSAVNAL 165  
 hVRF186 166 TPGPAAAAADAAASSVAKGGA\* 186  
 mvr186 166 TPGPAAAAADAAASSIAKGGA\* 186

FIG. 2. BESTFIT alignments of human and murine VRF protein isoforms. (A) mvr<sub>167</sub> and hVRF<sub>167</sub>. (B) mvr<sub>186</sub> and hVRF<sub>186</sub> from the point where the sequences diverge from the respective 167 amino acid isoforms. Amino acid identities are marked with vertical bars and conserved amino acids with colons. An arrow marks the predicted signal peptide cleavage site of human and murine VRF.

mvr167 -21 MSPLLRL..LLVALLQL..AR.TQAPVSQFDGPGSHQKVVFWIDVYARA 24  
 mvegf188 -26 MNFLLSWVHWTLLALLYLHAKWSQAAPTT.EGEQKSHEVIKFMVDVYQRS 23  
 mvr167 25 TCQPREVVVPLSMELMGNVVKQLVPSCVTVQRCGGCCPDDGLECVPTGQH 74  
 mvegf188 24 YCRPIETLVDFIQEYDIEYIFKPSVPLMRCAGCCNDEALECVPTSES 73  
 mvr167 75 QVRMQILMIQYPSSQ.LGEMSL E E H S Q C E C R P K K K E S A V K P D S P R ..... 118  
 mvegf188 74 NITMQIMRIKHQSQHIGEMSLFQHSRCECRPKKDRTPKPKSVRGKKGK 123  
 mvr167 119 .....ILCPPCTQRROR...PDPRTCRRCRRRRFLHCQGR 151  
 mvegf188 124 QKRKRKSRFSKWSVHCEPCSERRKHLFVQDPQTCKCSCKNTDS.RCKAR 172  
 mvr167 152 GLELNPDTCRCKPRK 167  
 mvegf188 173 QLELNERTCRCDKPRR 188

FIG. 3. BESTFIT alignment of mvr<sub>167</sub> and mvegf<sub>188</sub> [20] protein sequences. An arrow marks the signal peptide cleavage site of mvegf. Identical amino acids are indicated by vertical bars and conservative substitutions by colons. Numbering of amino acids is as described in the legend to Figure 1.



TABLE I  
Splice Junctions of the Murine *vrf* Gene

5' UTR*.....	Exon 1 >249bp	CCCAGgtacgtgct	Intron I	49bp
tttccacagGCCCC	Exon 2 43bp	GAAAGgtaataatag	Intron II	285bp
ctgcccacagTGGTG	Exon 3 197bp	TGCAGgtaccagggc	Intron III	196bp
ctgagcacagATCCT	Exon 4 74bp	TGCAGgtgccagcca	Intron IV	182bp
ctctttcagACCAA	Exon 5 36bp	GACAGgtgagtttt	Intron V	191bp
ctctctcagGGTTG	Exon 6 101bp		(no intron)	
CCCCTCCAGCCCCA	Exon 7 135bp	TGTAGgtaaggagtc	Intron VI	~220bp
cactccccagGTGCC	Exon 8 398bp	ATGGAAGACACTAAC		

Uppercase and lowercase letters denote exonic and intronic sequences, respectively. \*Indicates that the 5' end of exon I has not yet been determined.

and also maintains homology to *mveg* right through to the C-terminus (Fig. 3). The overall homology of *mvrf*<sub>167</sub> to *hVRF*<sub>167</sub> was 86% identity and 92% similarity respectively (Fig. 2). Likewise, homology between *mvrf*<sub>167</sub> and *mveg* [20] was 49% identity and 71% conservative amino acid substitution respectively (Fig. 3).

A canonical vertebrate polyadenylation signal (AATAAA) [21] was not present in the *vrf* cDNA, however, the closely matching sequence GATAAA is present at similar positions in both mouse and human *VRF* cDNAs (Fig. 1). In contrast to human *VRF*, murine *vrf* was found to contain an AC dinucleotide repeat at the extreme 3' end of the 3' UTR (nucleotide positions 997 to 1010, Fig. 1). Polymorphism of this repeat region was observed between some of the *vrf* cDNAs, with the number of dinucleotides varying from 7 to 11 (results not shown).

#### Genomic Characterisation of Murine *vrf*

Intron/exon boundaries (Table I) were mapped using primers which flanked sequences homologous to the corresponding human *VRF* boundaries [16]. Introns I, III, IV and VI of murine *vrf* (Table I, Fig. 4) were smaller than the *hVRF* intervening sequences [16]. There was complete concordance between the human *VRF* and murine *vrf* genes with respect to lengths of each of the exons. The complete genomic sequence was compiled from the 5' UTR of *vrf* through to intron VI, the largest intervening region (2.2kb), by sequencing amplified introns and cloned genomic portions of *vrf* (data not shown).

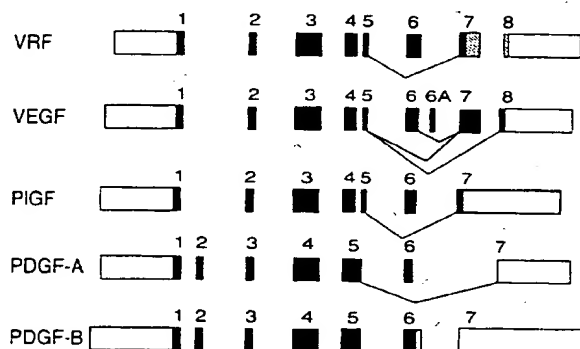


FIG. 4. Comparison of gene structure between *VRF* (a generic *VRF* gene is shown since the intron/exon organization of the mouse and human homologs is identical) and other members of the human *VEGF*/*PIGF*/*PDGF* gene family. Exons are represented by boxes. Protein coding regions and untranslated regions are shown by filled and open sections, respectively. The hatched region in *VRF* indicates the additional 3' UTR sequence formed by alternate splicing of the *VRF*<sub>167</sub> isoform. Potential alternate splice products of each gene are shown.

FIG. 5. Autoradiogram of a Northern blot of a murine *vrf* cDNA clone. A major tr.

Exons 6 and 7 are contiguous: strong sequence homology at the in the first half of Fig. 2B) suggests encodes a functional part of the

General intron/exon structure of the *VEGF* gene family and the murine *vrf* gene is very similar

Previous comparative mapping multiple endocrine neoplasia type the proximal segment of mouse to within 1Mb of the human *M*. the centromere of chromosome

#### Expression Studies of *vrf*

Northern analysis of RNA for expression appears to be ubiquitous in size (Fig. 5). This is somewhat major bands of 2.0 and 5.5kb in message presumably correspond thereof is most likely due to a

This work was supported by the National Ltd., and the Swedish Cancer Foundation

1. Sharkey, A. M., Charnock-Jones, I. 609-615.
2. Yan, Z., Weich, H. A., Bernart, W.
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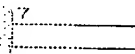
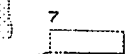
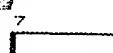
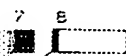
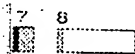
ne	
Intron I	495bp
Intron II	285bp
Intron III	190bp
Intron IV	182bp
Intron V	191bp
(no intron)	
Intron VI	720bp
AAC	

actively. \*Indicates that the 5' end of exon

C-terminus (Fig. 3). The overall similarity respectively (Fig. 2) 95% identity and 71% conservative

was not present in the *vrf* cDNA. at similar positions in both mouse murine *vrf* was found to contain in leotide positions 997 to 1010, Fig. some of the *vrf* cDNAs, with the (wn).

which flanked sequences homologous I, III, IV and VI of murine *vrf* sequences [16]. There was complete in respect to lengths of each of the 5' UTR of *vrf* through to intron VI. introns and cloned genomic por-



shown since the intron/exon organization in VEGF/PIGF/PDGF gene family. Exon shown by filled and open sections, respectively by alternate splicing of the VRF.

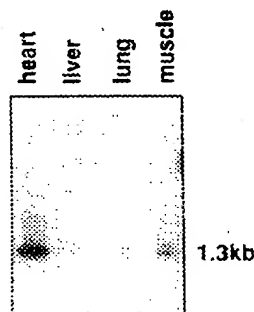


FIG. 5. Autoradiogram of a Northern blot of total RNA from various adult mouse tissues (as indicated) hybridized with murine *vrf* cDNA clone. A major transcript of 1.3kb was detected in all samples.

Exons 6 and 7 are contiguous in *vrf*, as has been found to occur in the human homolog [16]. The strong sequence homology at the amino acid level between exon 6 of *vrf* and human *VRF* (depicted in the first half of Fig. 2B) suggests that this sequence is not a retained intronic sequence but rather encodes a functional part of the *vrf*<sub>126</sub> isoform.

General intron/exon structure is conserved between the various members (VEGF, PIGF, VRF) of the VEGF gene family and therefore it is not surprising that the overall genomic organization of the murine *vrf* gene is very similar to these genes (Fig. 4).

Previous comparative mapping studies have shown that the region surrounding the human multiple endocrine neoplasia type 1 (*MEN1*) disease locus on chromosome 11q13 is syntenic with the proximal segment of mouse chromosome 19 [22]. Since we have mapped the human *VRF* gene to within 1Mb of the human *MEN1* locus [16], it is most likely that the murine *vrf* gene maps near the centromere of chromosome 19.

#### Expression Studies of *vrf*

Northern analysis of RNA from adult mouse tissues (muscle, heart, lung, and liver) showed that expression appears to be ubiquitous and occurs primarily as a major band of approximately 1.3kb in size (Fig. 5). This is somewhat different to the pattern observed for human *VRF* in which two major bands of 2.0 and 5.5kb have been identified in all tissues examined [16]. The 1.3kb murine message presumably corresponds to the shorter of the human transcripts and the size variation thereof is most likely due to a difference in the length of the respective 5' UTRs.

#### ACKNOWLEDGMENTS

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## A Novel Use for Coomassie Brilliant Blue G-250 Staining Procedure and Assay

Department of Pharmacology

The present study provides an alternative method for separating and quantifying proteins separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with coomassie brilliant blue (R-250) membranes. This method exhibited no shrinkage of gel, 2) it allowed as time of gel-drying procedure by a proteins with the same efficiency method is simple, economical and

Coomassie brilliant blue (R-250) thin layer chromatography protein concentrations (11-13). storage. However, gels shrink and gel-drying procedure is laborious replace the conventional method age problems. In this study, using gel drying process can be eliminated. Also, the described procedure h

**Materials.** Nitrocellulose membranes, glycine leupeptin, aprotinin, soybean trypsin inhibitor, purchased from Sigma Chemical Co. (St. Louis, MO). Sprague-Dawley rats were purchased from Harlan Sprague-Dawley.

**Preparation of rat brain protein extract.** Rats were sacrificed by decapitation. The total homogenate was solubilized with 0.05% (v/v) 2-mercaptoethanol, and electrophoresis as described below.

**Gel electrophoresis and electroblotting.** 7.5% acrylamide. Gels were stained with 0.1% Coomassie Brilliant Blue G-250 in 10% acetic acid and 20% methanol for 10-15 minutes. Stained gels were destained in 10% acetic acid and 20% methanol for 15 minutes and electroblotted onto nitrocellulose membranes were washed briefly in 10% acetic acid and 45% methanol and photographed using a Nikon camera.

**Amidoblack staining of membranes.** 10% acetic acid and 45% methanol and

<sup>1</sup> To whom correspondence should be addressed. Department of Pharmacology, 3500 car

COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

IN THE MATTER OF: Australian  
Patent Application 696764  
(73941/94). In the name of:  
Human Genome Sciences Inc:

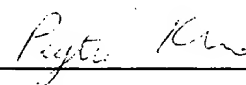
- and -

IN THE MATTER OF: Opposition  
thereto by Ludwig Institute for Cancer  
Research, under Section 59 of the  
Patents Act.

Annexure GBC-3

This is **Annexure GBC-3** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
\_\_\_\_\_  
Gary Baxter Cox

WITNESS:   
\_\_\_\_\_  
Patent Attorney PEYTEE KITCH

## Analysis of the Promoter Region of the Human VEGF-Related Factor Gene<sup>1</sup>

Ginters Silins,<sup>\*,2</sup> Sean Grimmond,<sup>\*</sup> Mark Egerton,<sup>†</sup> and Nicholas Hayward<sup>\*</sup>

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We have characterised the promoters of the human and murine *VRF* (vascular endothelial growth factor (VEGF) related factor) gene. A series of deletions were made of a 553-bp region 5' of the *VRF* initiation codon and were used in a luciferase reporter gene assay to determine the minimal promoter of the *VRF* gene. The region between base pairs -443 and -195 was sufficient to mediate transcription in lymphocytes and the region between -550 and -443 enhanced this promoter activity. Primer extension studies identified two regions of transcription initiation, both of which are preceded by Sp1, AP-2 and Egr-1 transcription factor binding sites. The *VRF* promoter is similar to *VEGF* in that it is associated with a CpG island, contains sites for Sp1 and AP-2, and lacks a TATA box. However, it has marked differences in that the promoter contains Egr-1 sites and lacks both hypoxia-inducible factor-1 and AP-1 sites. These data may indicate that expression of these two growth factors is regulated by different physiological stimuli. © 1997 Academic Press

We recently described the characterisation of a new member of the vascular endothelial cell growth factor (VEGF) gene family which we called *VRF* for VEGF-related growth factor [1, 2], but which is also known as *VEGFB* [3]. To date, this family of growth factors consists of *VEGFA/VEGF/VPF* [4, 5], *VEGFB/VRF* [1-3], *VEGFC/VRP* [6, 7], placenta growth factor [8], as well as the more distantly related platelet-derived growth factor (PDGFs) A and B [9].

<sup>1</sup>Sequences presented in this article have been submitted to the GENBANK database and appear under accession numbers: U80601 and U80602.

<sup>2</sup>To whom correspondence should be sent at Queensland Institute of Medical Research, P.O. Royal Brisbane Hospital, Herston 4029, Australia. Fax: (61) 7 3362 0107; E-mail: gints@qimr.edu.au.

Abbreviations: HIF-1 - hypoxia-inducible factor-1; PDGF - platelet derived growth factor; UTR - untranslated region; VEGF - vascular endothelial growth factor; *VRF* - human VEGF-related factor gene; *Vrf* - murine VEGF-related factor gene.

The intron-exon architecture of human *VRF* and murine *Vrf* [1, 2] are similar to that of *VEGFA*, and although only two alternately spliced forms of *VRF* have been identified (that give rise to proteins with different carboxyl tails), more isoforms are expected based on the gene's similarity to *VEGFA*. *VRF* has a wide tissue distribution in adults [1-3] and expression during mouse fetal development has been shown to be most abundant in heart, spinal cord, cerebral cortex and brown fat [10]. *VEGFA*, in comparison, is chiefly expressed in brain, kidney, liver, lung, spleen, as well as heart [11-13]. The expression pattern of these genes appears to be quite distinct, although specific roles for *VEGFA* and *VRF/VEGFB* in vasculogenic and angiogenic events have yet to be distinguished.

As a first step to understanding the regulation of *VRF* gene expression, we have characterised the promoters of the human (*VRF*) and murine (*Vrf*) genes. Reporter gene assays and primer extension studies were employed in identifying the minimal promoter region of *VRF*, and analysis of transcription factor binding motifs revealed that although the *VRF* and *VEGFA* promoters share common elements, there are also marked differences between the two promoters.

### MATERIALS AND METHODS

**Cloning, nucleotide sequencing and analysis.** The cloning of genomic fragments containing the human *VRF* [1] and murine *Vrf* genes [2] have been reported previously. A *Pst*I-restriction fragment containing part of the first coding exon of *VRF* plus the 5' flanking region (that included the 3' end of a novel gene manuscript in preparation) was subcloned from cosmid cCLGW4 [1]. Both nested deletions (Erase-a-base, Promega) and cloned restriction fragments of the region were sequenced on both strands as described previously [1, 2].

The cloning of a phage genomic clone ( $\lambda$ 121) containing the mouse *Vrf* gene has been reported previously [2]. Two *Nco*I-restriction fragments from  $\lambda$ 121 that collectively spanned exon 3 to the 5' flanking region were blunt-ended and cloned into the *Eco*RV site of pBlue-script II KS (Stratagene) and sequenced as described for *VRF*. The sequence data were compiled using MacVector 4.2.1 software (IBI-Kodak), ClustalW [14], CpGPlot [GCC, Wisconsin], Sigscan [15] and BLAST [16] analyses were conducted using the Australian National

Genome Information Service computer facility at the University of Sydney, Australia.

**Primer extension analysis.** The oligonucleotide 5' - TCC CGA GCC CTG GGT GCA G-3' was radiolabelled with [ $\gamma$ - $^{32}$ P]ATP (6000 Ci/mmol, Amersham) and T4 polynucleotide kinase (New England Biolabs). 5ng of labelled primer (1  $\mu$ l) were annealed to 60  $\mu$ g of total RNA in 10  $\mu$ l of annealing buffer consisting of 250mM KCl and 10mM Tris-HCl, pH 8.3. Total RNA was isolated from a lymphoblastoid cell line using an RNeasy Total RNA Kit (Qiagen). Annealing proceeded at 80°C for 5 min and then at 58°C for 45 min. First strand synthesis was allowed to proceed at 52°C for 45 min after the addition of 22.4  $\mu$ l of a reverse-transcription mix consisting of 3mM MgCl<sub>2</sub>, 47mM Tris-HCl, pH 8.3, 10mM dithiothreitol, 0.5mM dNTP mix and 200U of Superscript II RNaseH<sup>-</sup> reverse transcriptase (GIBCO, BRL). The RNA component was hydrolysed, the cDNA was precipitated, resuspended, resolved on an 8M urea/6% polyacrylamide gel and visualised by autoradiography. The primer extension product sizes were estimated by comparison to a sequencing ladder generated from pGEM-3zf(+) (Promega) with the pUC/M13 forward primer (mol DNA Sequencing System, Promega).

**Human VRF promoter constructs.** The most proximal 45bp of the first coding exon ending at a *Pst*I site (GENBANK accession number U43370) plus varying lengths of 5' flanking region derived from the human *Pst*I genomic clone were inserted into the polylinker of the pGL2-basic luciferase vector (Promega). Restriction sites within this genomic *Pst*I fragment were utilised in generating directional deletions of the region. These included, (in addition to the full-length *Pst*I fragment shown in Fig. 1), the *Sac*I site (position -550), the *Bst*XI site (position -443), the *Eag*I site (position -195) and the *Nco*I site (position -85).

**Transfections and measurements of luciferase activity.** 4  $\times$  10<sup>6</sup> EL4 T cells were suspended in 400  $\mu$ l RPMI 1640 tissue culture medium supplemented with 2mM glutamine and 20mM Hepes pH 7.2, and electroporated with 5  $\mu$ g of each luciferase reporter construct, or with 5  $\mu$ g empty pGL2-basic vector as a background control. Pulse conditions were 290V, 960  $\mu$ F. Electroporated cells were transferred to 10ml DMEM supplemented with 8% fetal calf serum, 2mM glutamine and 50  $\mu$ M  $\beta$ -mercaptoethanol, and grown for 24hr at 37°C in 5% CO<sub>2</sub> prior to harvesting. Harvested cells were washed in phosphate-buffered saline, and lysates prepared and assayed for luciferase activity using a Luciferase Assay Kit (Promega), according to the manufacturer's instructions. Luciferase activity was measured using a Packard Microplate Scintillation Counter. The protein content of each sample was measured by Bradford Assay (BioRad, Hercules CA), and values ranged from 42-68  $\mu$ g per sample. All data have been normalised to 50  $\mu$ g protein.

## RESULTS AND DISCUSSION

### Sequence Analysis of VRF

Sequence data for the open reading frame of *VRF* plus an adjacent 60bp of the 5' UTR have been reported previously [1]. We have extended the sequence of the 5' flanking region up to the transcribed region of a novel neighbouring gene as shown in Fig. 1. The program BLASTN identified nucleotide positions -629 to -553 as matching the 3' UTR of several cDNA entries from the expressed sequence tag database, complete with a polyadenylation signal (AATAAA) beginning at position -570 (manuscript in preparation). The promoter of *VRF* was therefore expected to reside downstream of this region.

The cloning and partial characterisation of *Vrf* has

been published by Townson et al. [2]. The previously reported 5' UTR of 189bp was extended an additional ~440bp up to the 3' UTR of the neighbouring gene (Fig. 1). (Note that 3 nucleotide discrepancies with respect to our previously described 5' UTR sequence have been corrected in the updated GENBANK entry U43836). Nucleotide sequences for the 5' flanking regions of the *VRF* and *Vrf* genes are aligned in Fig. 1 and show ~70% identity over this region. The sequences are G+C rich (~85% human, ~79% mouse) downstream of the 3' UTR of the neighbouring gene and contain a high frequency of CpG dinucleotides. Fig. 2 shows that the promoter of *VRF* coincides with a CpG island that spans the entire length of the region upstream of the initiation codon (Fig. 1). *VEGFA*, *PDGF A* and *B*, as well as numerous other growth factor genes (as discussed in [17]) also have G+C rich 5' UTRs.

Human *VRF* has three additional, apparently non-utilised ATG codons (positions -37, -83, -296) upstream of the reported translation start site (position +1). This start site was identified on the basis of sequence homology to *VEGFA* as well as matching the Kozak consensus sequence for vertebrate translation initiation sites [1]. The three non-utilised ATG codons are also conserved in *Vrf* (positions -37, -83, -306) and the latter two are not in-frame with the translation initiation site in either organism. The ATG codon at position -296 in *VRF* is also out of frame, but translation from the equivalent ATG codon from *Vrf* (position -306) would extend the reported murine protein by an additional 102 amino acids [2]. However, primer extension studies with *VRF* (discussed below) predict that this ATG codon overlaps one region of transcription initiation and is therefore unlikely to be present in a large proportion of *VRF* transcripts.

The possible function of these upstream ATG codons is unknown, however, they may play a role in translational control as has been suggested in the case of the genes encoding the A- and B- chains of PDGF which have also been reported to each have three additional ATG codons [18]. Similarly, a second conserved ATG codon appears within the 5' UTR of human [17], mouse [19] and rat [20] *VEGFA*.

### Reporter Gene Assays

In order to define the proximal promoter of *VRF*, restriction sites within the 5' flanking region (shown in Fig. 1) were used to generate successive deletions from within the 3' UTR of the neighbouring gene towards the transcription start site of *VRF*. These restriction fragments were directionally cloned into the promoterless vector pGL2, transiently transfected into lymphocytes, and assayed for luciferase activity. The results from each of three experiments showed a similar trend (Fig. 3), with the *Pst*I construct (Fig. 3, A-*Pst*I) that spans the coding region of *VRF* exon 1 to the

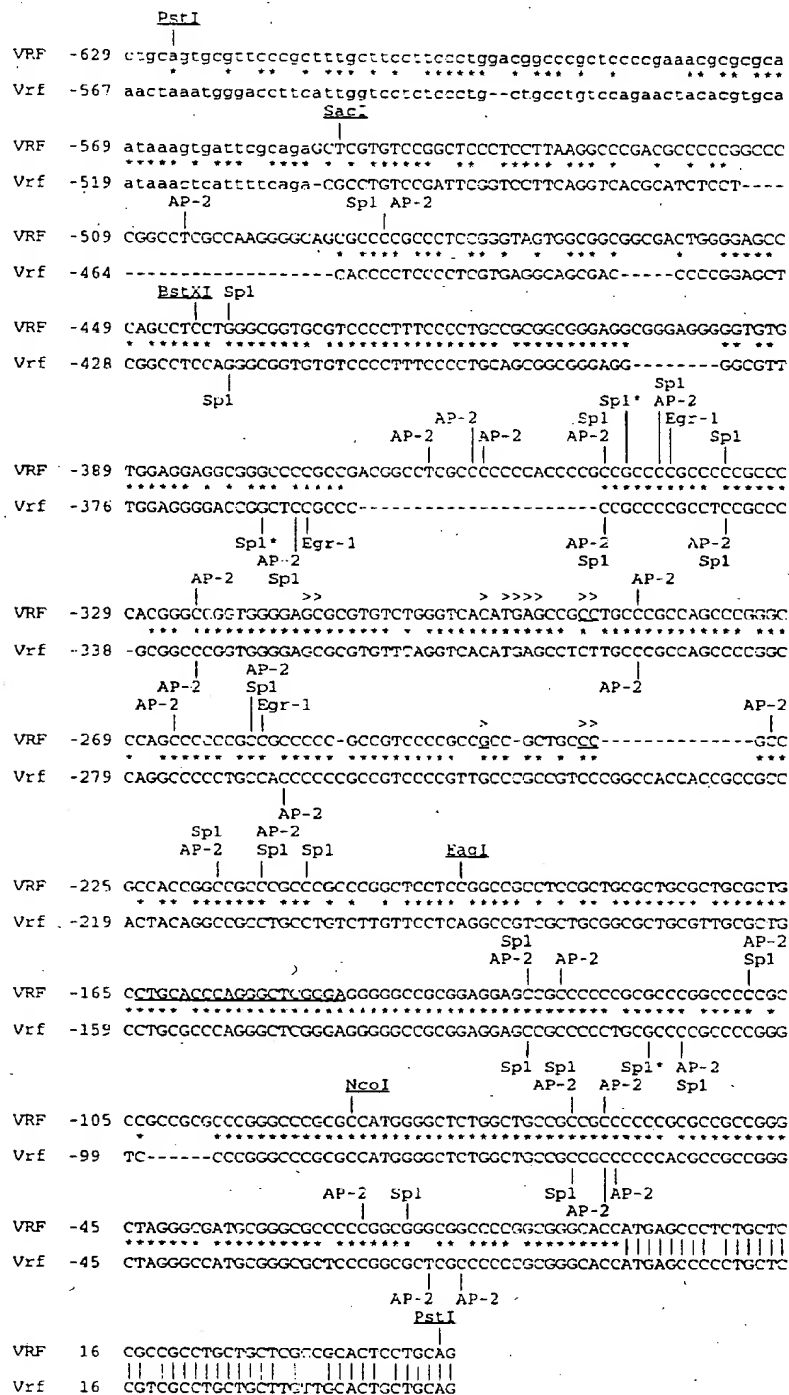


FIG. 1. Nucleotide sequence alignment of human (*VRF*) and murine (*Vrf*) *VRF* 5' flanking regions using the program ClustalW, where an asterisk denotes a conserved nucleotide position and a vertical line indicates conservation within the open reading frame. The *VRF* start codon is designated position +1. The 3' UTR of the neighbouring gene is represented in lower case lettering. Restriction sites used in generating the promoter constructs are labelled. Positions of the transcription start sites as determined by primer extension studies are shown by the symbol ">" and the major sites are underlined. The position of the oligonucleotide used for primer extension is underlined. Starting positions of the consensus binding sites for the transcription factors Sp1, "strong" Sp1 (\*), AP-2 and Egr-1 are labelled and described in the text.

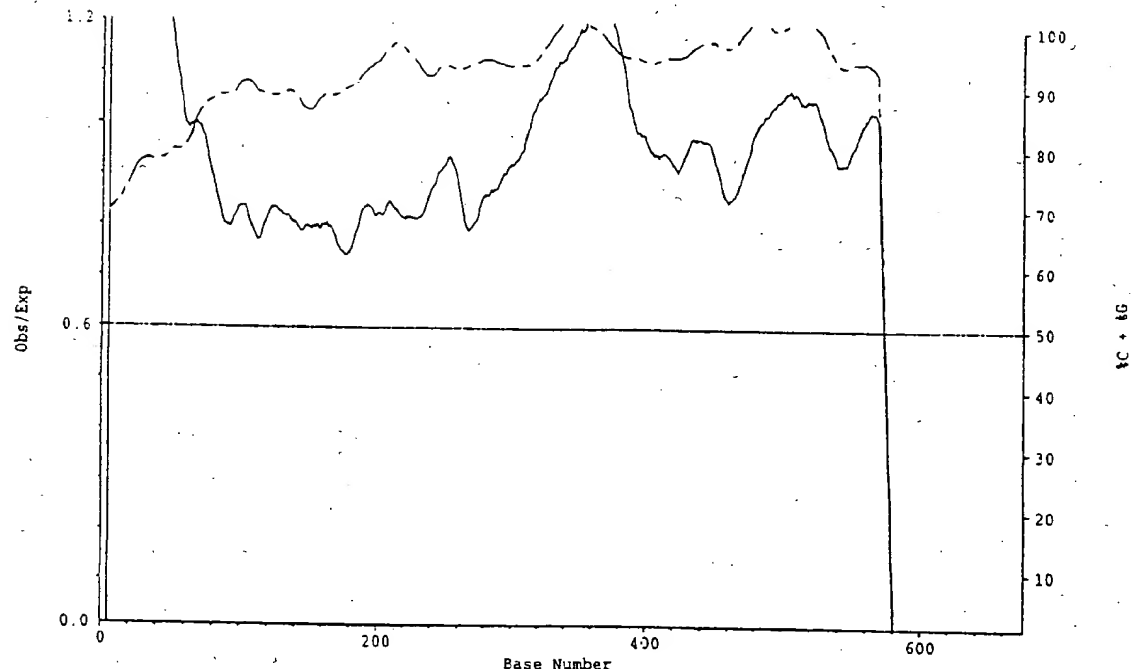


FIG. 2. CpGPlot of the *VRF* promoter region (based on the sequence of Fig. 1). Plot of Obs/Exp CpG dinucleotide (solid line) and %G+C (broken line) against the position in the nucleotide sequence. The CpG island of the *VRF* gene meets the criteria described by Larsson et al. [25] (>200bp region; moving average %G+C > 50; moving Obs/Exp CpG > 0.6).

3' UTR of the neighbouring gene, producing a 1.6 to 9.6-fold increase in luciferase activity over background levels. The *SacI* deletion construct (Fig. 3, B-*SacI*) produced the highest promoter activity (3.8 to 12.9-fold increase), presumably because the polyadenylation signal (and possibly other *cis*-acting elements involved in transcription termination) from the neighbouring gene had been removed. The *BstXI* deletion construct (Fig. 3, C-*BstXI*) produced a lower (28-47%) promoter activity compared to the *SacI* construct, but roughly equivalent to the activity observed for the *PstI* construct. The *EagI* and *NcoI* deletion constructs (Fig. 3, D-*EagI*, E-*NcoI*) had luciferase activities at background levels. We therefore conclude that the 248bp region between *BstXI* (position -443) and *EagI* (position -195) is sufficient to promote basal transcription of the *VRF* gene in lymphocytes, and that the 108-bp region upstream of this (up to the *SacI* site at position -50) is necessary for maximal basal activity.

#### Primer Extension Analysis, Structural Analyses of the Promoter Region

Primer extension analysis, using an antisense oligonucleotide designed to anneal downstream (positions -146 to -164) of the minimal promoter region was employed to identify the 5' end of the human *VRF* transcript. These assays consistently demon-

strated the *VRF* gene to have two major regions of transcription initiation, although additional start sites were observed on longer exposure of the autoradiographs (Fig. 4). The first region of transcription initiation consisted of a cluster of sites between positions -229 and -238 (Fig. 1). A second and more frequently used cluster of transcription initiating sites appeared between positions -313 to -287, suggesting a maximum 5' UTR length of 313bp that is highly conserved (~84%) in the murine gene. The *VRF* 5' UTR is therefore much shorter in length than that of the *VEGFA* gene [17].

The mouse and human *VRF* promoter regions were scanned for consensus transcription factor binding sites using the program Sigscan. Sites that potentially play a role in the regulation of *VRF* gene expression, or that have been reported for *VEGFA* [19, 20], are shown in Fig. 1. The region of transcription initiation between positions -313 and -287, preceded by multiple consensus sites for Sp1 (5'-CCGCCC-3' or its complement [21, 22]) and AP-2 (5'-T/CCC/GCCA/CNC/GC/GC/G-3' or its complement [23]), as well as a single site for Egr-1 (5'-GGG GGGCG-3' or its complement [24]). Sites for Sp1, AP-2 and Egr-1 are also found clustered in a similar position in the mouse gene. The same combination of sites (in the human but not in the mouse gene) precede the downstream region of transcription initiation.



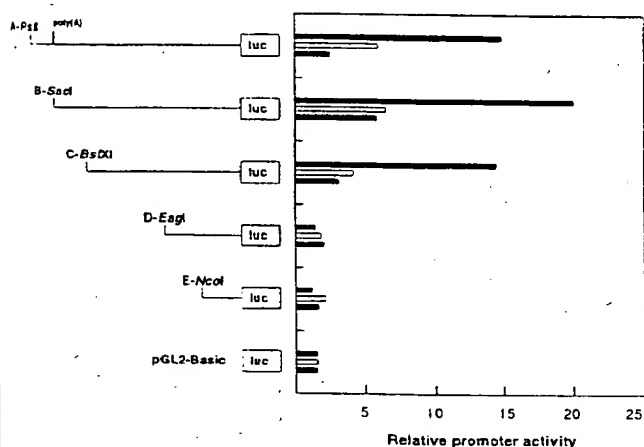


FIG. 3. Determination of the *VRF* minimal promoter region. Sections of the *VRF* 5' flanking region were cloned into the pGL2 luciferase vector and assayed for their ability to induce luciferase activity in EL4 T cells. The position of the poly(A) tail for the neighbouring gene is shown in construct A and the exact locations of the restriction sites are shown in Fig. 1. Relative promoter activities for the constructs based on triplicate experiments are represented by different shadings.

tion (positions -265 to -209). Examination of the highly conserved *Bst*XI to *Eag*I region of *VRF* and *Vrf* revealed additional Sp1 and AP-2 sites. The Sp1 site at position -344 in the human gene (position -364 in mouse) is also a perfect match to the "strong" Sp1 consensus 5'-G/AC/TC/TCGGCCCC/A-3' that has been reported for some of the Sp1 sites within the *VEGFA* promoter [17]. Indeed, the promoters of *VEGFA* and *VRF* appear to be organised similarly with respect to Sp1 and AP-2 sites, and both promoters are associated with a CpG island (data not shown for *VEGFA*), which typically encompass the transcription start sites of housekeeping and widely expressed genes [25]. The presence of AP-2 sites is consistent with the observed expression patterns of these genes in tissues of neural origin [10, 26]. As with *VEGFA* [17, 19, 20], a consensus TATA box is absent from the *VRF* proximal promoter.

The promoter of *VRF* also shows potentially important differences to the promoter of *VEGFA*, for example, more than one region of transcription initiation appears to be utilised by *VRF*, and the human and mouse *VEGFA* promoter regions also contain CCAAT boxes upstream of their transcription start sites [17, 19]. Although multiple AP-1-like binding sites were reported for human *VEGFA*, only one consensus AP-1 site, located near to the hypoxia element, is conserved between human, mouse and rat sequences [17, 19, 20]. There is an absence of consensus AP-1 sites (5'-TGANTC/AA-3' [27]) and sequences resembling the hypoxia-inducible factor

(HIF-1: 5'-G/C/TACGTGCG/T-3' [28]; *VEGFA* HIF-1 match: 5'-TACGTGGG-3' [20]) in the *VRF* promoter region. However, it is possible that other hypoxia-responsive sequence elements, not related to HIF-1, influence transcription, potentially also from other regions of the gene (such as the 3' end) as has been reported for *VEGFA* [29].

The human *VRF* promoter has two occurrences of overlapping Egr-1 and Sp1 sites, upstream of the two major regions of transcription initiation. The replacement of Sp1 by Egr-1 at an overlapping site can lead to inducible gene expression, as observed for the *PDGF A* and *B* genes [30, 31]. Overlapping Sp1 and Egr-1 sites, however, are not present in the promoter region of *VEGFA* [17, 19, 20]. Studies of the *PDGF A* and *B* promoter regions infer that *VRF* may utilise novel transcription factor binding sites as well as perhaps

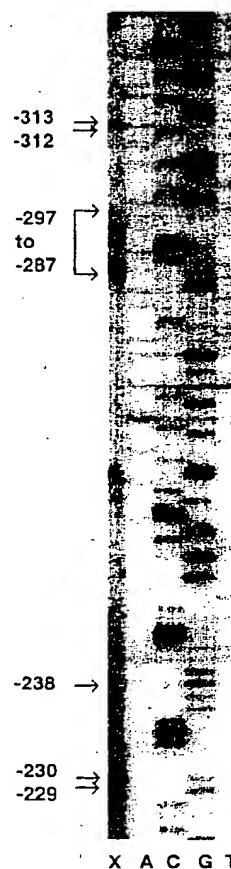


FIG. 4. Mapping of the *VRF* transcription start site using primer extension analysis. Primer extension products (lane labelled X) were generated from lymphoblastoid cell line total RNA using the oligonucleotide shown in Fig. 1 and the sites of transcription initiation are labelled with arrows. The size standard was generated from pGEM-3z(+) using the pUC/M13 forward primer (lanes G, A, T and C).

non-consensus sites [32, 33]. A candidate region likely to contain these sites is positioned between -404 and -455 of *VRF*, as the region is highly conserved in mouse and precedes the upstream region of transcription initiation.

The findings reported in this paper provide the first insight into the organisation of the *VRF* proximal promoter and address possible differences with respect to the promoter of *VEGFA*. Further studies will be aimed at elucidating the sequence elements important for inducible gene expression, in an effort to understand the role of *VRF* in vasculogenic and angiogenic events.

## ACKNOWLEDGMENTS

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- and -

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Thirteenth day of December 2000.

*Gary Baxter Cox*  
Gary Baxter Cox

WITNESS: *Peetee Kitta*  
Patent Attorney *PEYTEE Kitta*

# Circulation Research: Journal of the American Heart Association

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## Mice Lacking the Vascular Endothelial Growth Factor-B Gene (*Vegfb*) Have Smaller Hearts, Dysfunctional Coronary Vasculature, and Impaired Recovery From Cardiac Ischemia

[Ultrarapid Communications]

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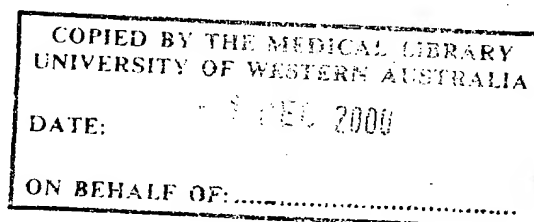
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## Abstract

**Abstract:** Vascular endothelial growth factor-B (VEGF-B) is closely related to VEGF-A, an effector of blood vessel growth during development and disease and a strong candidate for angiogenic therapies. To further study the *in vivo* function of VEGF-B, we have generated *Vegfb* knockout mice (*Vegfb*<sup>-/-</sup>). Unlike *Vegfa* knockout mice, which die during embryogenesis, *Vegfb*<sup>-/-</sup> mice are healthy and fertile. Despite appearing overtly normal, *Vegfb*<sup>-/-</sup> hearts are reduced in size and display vascular dysfunction after coronary occlusion and impaired recovery from experimentally induced myocardial ischemia. These findings reveal a role for VEGF-B in the development or function of coronary vasculature and suggest potential clinical use in therapeutic angiogenesis. The full text of this article is available at <http://www.circresaha.org>.

Vascular endothelial growth factor-B (VEGF-B) <sup>1,3</sup> is a secreted growth factor that has strong sequence homology with VEGF-A, a primary regulator of angiogenesis in development, corpus luteum formation, wound healing, and cancer. <sup>4</sup> VEGF-B can form stable heterodimers with VEGF-A <sup>3</sup> and is generally coexpressed with VEGF-A. <sup>5,6</sup> VEGF-B can bind to two of the VEGF-A receptors, VEGFR-1 <sup>7</sup> and neuropilin-1, <sup>8</sup> suggesting that it may regulate the bioavailability and/or action of VEGF-A. <sup>5</sup> Although VEGF-B has been reported to behave as an endothelial cell mitogen, <sup>2</sup> part of the mitogenic activity reported may be due to VEGF-B/VEGF-A heterodimers. <sup>5</sup>

Several mouse models have been generated by gene knockout technology where the genes encoding *Vegf*-A or its receptors have been mutated. Both *Vegfa*<sup>-/-</sup> and *Vegfa*<sup>+/-</sup> mice are unable to survive to term due to a general impairment of blood vessel formation in the early embryo. <sup>9,10</sup> *Vegfa*<sup>120/120</sup> mice, where only two of the three major *Vegf*-A isoforms have been knocked out, die postnatally after cardiac failure due to widespread myocardial ischemia. <sup>11</sup> *Vegfr1*<sup>-/-</sup> mice die as embryos due to defects in angiogenesis, <sup>12</sup> but partial knockout mice, where only the tyrosine kinase-encoding portion of the *Vegfr1* gene is deleted, develop normal vasculature. <sup>13</sup>

To study the *in vivo* role of *Vegf*-B, we have generated a knockout mouse line and found that, unlike the *Vegf*-A-related knockouts, *Vegfb*<sup>-/-</sup> mice appear outwardly normal and fertile. Because *Vegfb* transcripts are expressed predominantly in the heart during murine embryogenesis and adult life, <sup>1,14-16</sup> suggesting a specific role for *Vegf*-B during cardiac development, we have concentrated on studying the cardiac phenotype in these mice. *Vegfb*<sup>-/-</sup> hearts are reduced in size compared with hearts of *Vegfb*<sup>+/-</sup> littermates and display clinical symptoms of impaired recovery from experimentally induced ischemia. The results suggest an essential role for *Vegf*-B in establishment of a fully functional coronary vasculature and highlight the potential of this cytokine for application in the emerging field of therapeutic angiogenesis.

## Materials and Methods

### Generation of *Vegfb*<sup>+/-</sup> Mice

All mice used for the present study were supplied by the Animal Resources Centre (Western Australia), and their treatment was in accordance with the National Health and Medical Research Council (NH&MRC) guidelines for the care of experimental animals.

Targeted inactivation of the *Vegfb* gene was achieved by replacing exons 3 to 7 (Figure 1a) with a promoter-less [*beta*]-*geo* cassette. The [*beta*]-*geo* gene was preceded by an internal ribosomal entry site to give cap-independent translation of [*beta*]-*geo*. <sup>17</sup> Targeted 129/SvJ ES cells (C1368) were injected into C57BL/6J blastocysts to produce chimeras. Progeny of germline-transmitting chimeras were genotyped by polymerase chain reaction (PCR) amplification of tail-tip DNA using PCR1, 5'-TTT GAT GGC CCC AGC CAC-3'; PCR2, 5'-CCC CCA GCT GAC TGC TCG-3'; and PCR3, 5'-CTA GTG GAT CCC CCG GGC-

3' (Figure 1b).

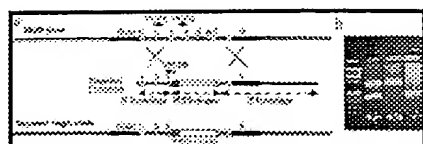


Figure 1. a, Diagram of the murine *Vegfb* gene (top), the targeting construct used to generate a *Vegfb*<sup>+/+</sup> mouse (middle), and the final targeted locus (bottom). The exons of the *Vegfb* gene are shown as numbered boxes with the open reading frame as open boxes. The location and orientation of the PCR primers used to genotype mice are shown as PCR1, PCR2, and PCR3. b, Genotyping PCR on *Vegfb*<sup>+/+</sup>, *Vegfb*<sup>+/-</sup>, and *Vegfb*<sup>-/-</sup> DNA, using the primers shown in panel a.

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## [beta]-Gal Staining and Immunohistochemistry

Frozen sections and whole embryos were stained for [beta]-Gal as described.<sup>18</sup> For quantitation of capillary density, transverse sections of the left ventricle (LV) were cut at comparable levels in *Vegfb*<sup>+/+</sup> and *Vegfb*<sup>-/-</sup> P30 hearts (30 days postpartum) (4 hearts each), immunostained with anti-PECAM-1 (clone M13, Pharmingen), and the capillaries counted using ImagePlus software on 7 randomly chosen fields ( $\times 40$  magnification, [almost equal to] 0.06 mm<sup>2</sup> per field) in the epicardial, endocardial, and midmyocardial portion of the LV. Because no difference between genotypes was found within each portion, capillary density data were averaged for each heart. Coronary vessels were counted as anti-smooth muscle [alpha]-actin (FITC conjugated, clone 1A4, Sigma)-stained vessels in whole sections.

## Heart Weight and LV Thickness

*Vegfb*<sup>+/+</sup>, *Vegfb*<sup>+/-</sup>, and *Vegfb*<sup>-/-</sup> mice of either 129/SvJ or C57BL/6J $\times$ 129/SvJ background were weighed. After dissection, the hearts were trimmed of surrounding tissue and weighed. A subset of the P25 hearts was fixed in formalin and microdissected to obtain a similar angle of section. LV thickness was measured on sections with a stage micrometer (n=10 *Vegfb*<sup>+/+</sup> hearts; n=16 *Vegfb*<sup>+/-</sup> hearts; n=14 *Vegfb*<sup>-/-</sup> hearts).

## Langendorff Perfusion

Hearts were isolated from mice anesthetized with 60 mg/kg sodium pentobarbital. *Vegfb*<sup>+/+</sup> (161 $\pm$ 7 mg wet heart weight [WHW], n=15), *Vegfb*<sup>+/-</sup> (152 $\pm$ 6 mg WHW, n=14), and *Vegfb*<sup>-/-</sup> mice (155 $\pm$ 7 mg WHW, n=16) hearts were perfused in the Langendorff mode as described.

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For ischemia, baseline measurements were recorded from *Vegfb*<sup>+/+</sup> (n=8), *Vegfb*<sup>+/-</sup> (n=8), and *Vegfb*<sup>-/-</sup> hearts (n=8) after 30 minutes of stabilization. Global normothermic ischemia was initiated for 20 minutes before 30 minutes of aerobic reperfusion. To examine reactive hyperemia, a subset of hearts (n=7 for *Vegfb*<sup>+/+</sup>, n=6 for *Vegfb*<sup>+/-</sup>, and n=8 for *Vegfb*<sup>-/-</sup>) was perfused as described above and after stabilization was subjected to a single 20-second period of zero flow followed by reperfusion at 90 mm Hg perfusion pressure. The coronary flow response was recorded, peak hyperemic flows were measured in individual experiments, and percentage of flow-debt repayment over the initial 60 seconds of reperfusion was calculated as follows: MATH where total coronary flows were measured in mL/g and were calculated by digital integration of coronary flow for the 60 seconds before and 60 seconds after occlusion using the Chart V3.5.6 program (AD Instruments, Castle Hill, Australia), and flow-debt was calculated as basal coronary flow (mL/60 seconds/g) $\times$ 20 seconds of occlusion.

The equation is: 
$$\text{Flow-debt repayment (\%)} = \frac{\text{Total flow (mL/g)} - \text{Flow before occlusion (mL/g)} \times 20}{\text{Flow before occlusion (mL/g)}} \times 100$$

Equation 1

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## Statistical Analyses

Body/heart weight, LV thickness, and capillary density data were analyzed using unpaired Student's *t* tests. Body/heart weight data were also analyzed using the generalized estimation equation.<sup>20</sup> Hyperemia data were analyzed via one-way ANOVA and functional parameters by two-way ANOVA for repeated measures. Where significant effects were detected, the

Tukey's HSD post hoc test was used. In all tests, significance was accepted at  $P < 0.05$ .

An expanded Materials and Methods section is available online at <http://www.circresaha.org>.

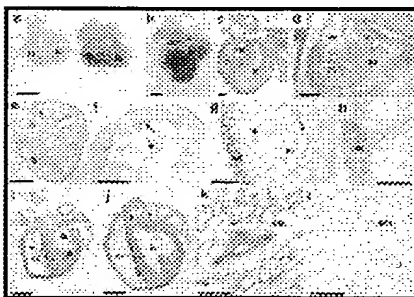
## Results

### Generation of the *Vegfb*<sup>-/-</sup> Mice

The *Vegfb* knockout mice generated with the modified *Vegfb* locus shown in Figure 1a were produced in normal mendelian ratios, were healthy and fertile, and did not display any overt phenotype. The genotype of mice was determined by PCR amplification of tail-tip DNA from P10 pups (Figure 1b). Rather than producing Vegf-B, this modified locus results in [beta]-Gal expression under the control of the endogenous *Vegfb* promoter (herein referred to as Vegf-B/[beta]-Gal). Because the *Vegfb*<sup>+/-</sup> mice generated in this manner had no obvious developmental defects, we assumed that Vegf-B/[beta]-Gal expression in these mice accurately reflects the endogenous *Vegfb* expression pattern.

### Cardiac Vegf-B/[beta]-Gal Expression Pattern in the *Vegfb*<sup>+/-</sup> Mouse

Using [beta]-Gal staining, Vegf-B/[beta]-Gal expression was first detected in the heart at E10.5 (embryonic day 10.5), it became prominent at E12.5 (Figure 2a) and further increased thereafter (Figure 2b). Throughout development, Vegf-B/[beta]-Gal expression appeared to be restricted to the myocardium (Figures 2c through 2g) and subepicardium (Figures 2g and 2h) and remained undetectable in endothelial cells, including those of the endocardium and coronary endothelium. Endocardial derivatives, such as the valve leaflets were always devoid of Vegf-B/[beta]-Gal expression. During development, the highest concentration of Vegf-B/[beta]-Gal-expressing cells was seen in the right ventricular myocardium and right aspect of the interventricular septum (Figures 2a through 2c). Lower Vegf-B/[beta]-Gal expression was detectable in the LV (Figures 2a and 2c) and the right atrial appendage (Figure 2d). The lowest expression was found in the atrial wall (Figures 2c, 2d, and 2f), where coronary angiogenesis is less conspicuous (Figures 2e and 2f). Within the right ventricle (RV), Vegf-B/[beta]-Gal staining was prevalent in the trabeculations (Figure 2d). The intensity of Vegf-B/[beta]-Gal expression increased further in the neonate heart (Figures 2i and 2j) in correlation with the massive early postnatal coronary capillary and vessel growth.<sup>11</sup> The prevalence of Vegf-B/[beta]-Gal expression switches from the RV to the LV in the early neonatal period (Figures 2i and 2j) reflects the predominant early postnatal capillarization of this chamber.<sup>21</sup> In the juvenile heart, the ventricular prevalence of expression is lost, and the density of Vegf-B/[beta]-Gal-expressing cells is similar in the ventricles and the atria (data not shown).



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Figure 2. Vegf-B/[beta]-Gal localization in the heart during development and after birth. a, Whole-mount E12.5 Vegf-B/[beta]-Gal-stained *Vegfb*<sup>+/-</sup> (right) and *Vegfb*<sup>-/-</sup> (left) hearts showing higher expression in the RV. b through d, Whole mount and sections of Vegf-B/[beta]-Gal-stained E15.5 hearts. b, *Vegfb*<sup>+/-</sup> whole mount. Vegf-B/[beta]-Gal levels are very low in the aorta and pulmonary trunk. c, Longitudinal section of the *Vegfb*<sup>+/-</sup> heart shown in panel b. Note higher levels of Vegf-B/[beta]-Gal expression (staining blue) in the RV and right side of the interventricular septum (\*) than the LV. d, Sagittal section through the RV. Note expression in the trabeculations of the ventricle and absence in the atria. e through h, E17.5 hearts. e, Longitudinal heart section stained for PECAM-1 (staining brown). The rectangles represent the regions in panels f and g. f and g, Vegf-B/[beta]-Gal and anti-PECAM-1 double labeling. f, Atrial region. The endocardium (arrows) is stained with anti-PECAM-1, but no capillaries have formed in this region. g, RV. Anti-PECAM-1 stains the endocardium (arrow) and the endothelium of numerous capillaries (arrowhead) surrounded by Vegf-B/[beta]-Gal-expressing cardiomyocytes in the myocardium and subepicardium. h, Higher magnification of the subepicardial region stained for Vegf-B/[beta]-Gal expression showing several Vegf-B/[beta]-Gal-positive cells. i through k, P3 *Vegfb*<sup>+/-</sup> mouse hearts. i and j, Vegf-B/[beta]-Gal staining of transverse heart sections. j, Section through the atrioventricular transition. Note prominent staining in the LV. j, Section through the ventricular region. Note the absence of staining around the coronary vessels (arrowheads). k and l, Contiguous sections through a coronary arteriole stained for PECAM-1 (j) and Vegf-B/[beta]-Gal (k). Vegf-B/[beta]-Gal-expressing cardiomyocytes surround the capillaries, but Vegf-B/[beta]-Gal is undetectable in the endothelium and smooth muscle layers surrounding the artery. rv indicates right ventricle; lv, left ventricle; \*, interventricular septum; ra, right atrium; aa, atrial appendage; m, myocardium; se, subepicardium; and p, pulmonary trunk. Bar=500  $\mu$ m (a, b, d, e, i, and j) and 100  $\mu$ m (c, f, g, h, k, and l).

The great arteries in the heart expressed low levels of Vegf-B/[beta]-Gal at all stages of development (eg, Figure 2b) and in juvenile mice (data not shown). Vegf-B/[beta]-Gal was undetectable in the tunica intima and media of coronary vessels (Figures 2i, arrowheads, and 2k and 2l), although we found Vegf-B/[beta]-Gal expression in other vessels in the body (eg, the intralobar component of the pulmonary arteries) (data not shown).

### Postnatal Heart Growth in *Vegfb*<sup>-/-</sup> Mice

Although histological examination of all organs revealed no differences between genotypes, *Vegfb*<sup>-/-</sup> hearts frequently appeared marginally smaller than their *Vegfb*<sup>+/+</sup> and *Vegfb*<sup>+/-</sup> littermates (Figure 3a). We recorded the total body and heart weight of 122 animals including male and female *Vegfb*<sup>+/+</sup>, *Vegfb*<sup>+/-</sup>, and *Vegfb*<sup>-/-</sup> mice at several ages between P3 and P91. These mice were grouped as P3 to P9 mice (*Vegfb*<sup>+/+</sup>, n=15; *Vegfb*<sup>+/-</sup>, n=20; and *Vegfb*<sup>-/-</sup>, n=13) and P25 or older (*Vegfb*<sup>+/+</sup>, n=20; *Vegfb*<sup>+/-</sup>, n=28; and *Vegfb*<sup>-/-</sup>, n=27). We found no consistent genotype-dependent decrease in body weight; however, heart weight was always reduced in *Vegfb*<sup>-/-</sup> mice. To account for the inherent interlitter and intralitter variability in body weight, due to sex, age, and genetic background, we used heart/body ratios to display the results. Although we found no significant difference in heart/body ratio in relation to sex or genetic background, statistical analysis revealed a dramatic increase in heart/body ratio from P3-9 to P25 (or older) in all animals regardless of genotype (Figure 3b). There were no differences in percentage of heart/body weight ratios among genotypes in P3-9 mice (*Vegfb*<sup>+/+</sup>, 0.64±0.02; *Vegfb*<sup>+/-</sup>, 0.59±0.03; and *Vegfb*<sup>-/-</sup>, 0.66±0.03), but we found a significant (*P*<0.05) decrease in percentage of heart/body weight ratio in P25 (or older) *Vegfb*<sup>-/-</sup> (0.78±0.02) mice compared with *Vegfb*<sup>+/+</sup> (0.87±0.04) and *Vegfb*<sup>+/-</sup> (0.89±0.02) mice (Figure 3b). When familial (litter/parents) correlation among mice was taken into account, this significant difference remained (data not shown). No significant difference was found between *Vegfb*<sup>+/+</sup> and *Vegfb*<sup>+/-</sup> mice at any stage.

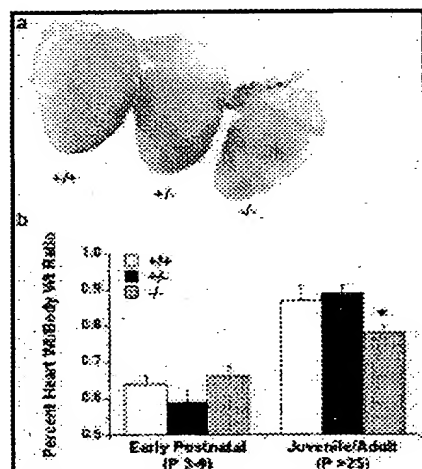


Figure 3. Reduced postnatal heart size in *Vegfb*<sup>-/-</sup> hearts. a, Appearance of P25 *Vegfb*<sup>+/+</sup>, *Vegfb*<sup>+/-</sup>, and *Vegfb*<sup>-/-</sup> hearts from same-sex littermates, illustrating a slightly reduced *Vegfb*<sup>-/-</sup> heart size. b, Percentage of body/weight ratio is significantly reduced in the juvenile (>P25) *Vegfb*<sup>-/-</sup> mice but not in early postnatal mice (P3-9), indicating the impaired growth of the *Vegfb*<sup>-/-</sup> hearts in the first few weeks after birth. Values are mean±SEM. \**P*<0.05 vs *Vegfb*<sup>+/+</sup> mice; *P*<0.01 vs *Vegfb*<sup>+/-</sup> mice.

The dramatic increase in heart weight during the first few weeks after birth is well documented and appears to be mainly due to massive growth of the coronary capillaries and vessels, but cardiomyocyte proliferation and hypertrophy also contribute. 22 No difference in the size of myocardial cells was found in histological sections in *Vegfb*<sup>+/+</sup>, *Vegfb*<sup>+/-</sup>, or *Vegfb*<sup>-/-</sup> hearts (data not shown). LV thickness was significantly decreased in P25 *Vegfb*<sup>-/-</sup> (0.80±0.03 mm, \**n*=14) compared with *Vegfb*<sup>+/+</sup> (0.89±0.03 mm, *n*=10) and *Vegfb*<sup>+/-</sup> (0.91±0.02 mm, *n*=16) hearts (\**P*=0.059 versus *Vegfb*<sup>+/+</sup> and *P*<0.05 versus *Vegfb*<sup>+/-</sup>). Analysis of capillary density using standard morphometric measures found no significant differences between P30 *Vegfb*<sup>+/+</sup> (2321±255 capillaries/mm<sup>2</sup>) and *Vegfb*<sup>-/-</sup> (2334±253 capillaries/mm<sup>2</sup>) hearts. Vessel density measures in adjacent heart sections also showed no differences between *Vegfb*<sup>+/+</sup> (270±10 vessels/section) and *Vegfb*<sup>-/-</sup> (275±14 vessels/section) hearts.



## Reactive Hyperemic Responses in *Vegfb*<sup>+/+</sup>, *Vegfb*<sup>+/-</sup>, and *Vegfb*<sup>-/-</sup> Hearts

Baseline contractile function and coronary flow were equivalent in Langendorff-perfused hearts from all three groups under normoxic conditions (see Table online, <http://www.circresaha.org>). To test whether alterations in vascular function would be more evident during active responses to modified myocardial O<sub>2</sub> delivery, we exposed hearts to transient (20 seconds) coronary occlusion and studied the hyperemic response on reperfusion. The reactive hyperemic responses differed subtly between groups (Figure 4). Although peak hyperemic flow was comparable in all three groups of hearts (32 to 36 mL · min<sup>-1</sup> · g<sup>-1</sup>) (Figure 4a), overall flow-debt repayment during the initial 60 seconds of reperfusion (during which flow recovered to preocclusion levels) was significantly lower in *Vegfb*<sup>-/-</sup> mice ([almost equal to]60%) versus the other two groups ([almost equal to]100%) (Figure 4b). There were no differences in repayment between *Vegfb*<sup>+/+</sup> and *Vegfb*<sup>+/-</sup> hearts. These findings indicate that the functional status of the coronary vasculature is impaired in *Vegfb*<sup>-/-</sup> mice.

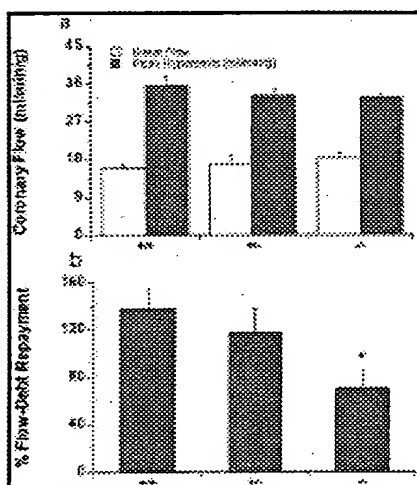


Figure 4. Reactive hyperemic responses to 20-second coronary occlusion in Langendorff-perfused *Vegfb*<sup>+/+</sup>, *Vegfb*<sup>+/-</sup>, and *Vegfb*<sup>-/-</sup> hearts. a, Baseline and peak hyperemic flows. b, Flow-debt repayments over the initial 60 seconds of reperfusion. Values are mean±SEM. \**P* < 0.05 vs *Vegfb*<sup>+/+</sup> hearts.

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## Responses to Ischemia-Reperfusion in *Vegfb*<sup>+/+</sup>, *Vegfb*<sup>+/-</sup>, and *Vegfb*<sup>-/-</sup> Hearts

As noted, baseline functional parameters were comparable in hearts from *Vegfb*<sup>+/+</sup>, *Vegfb*<sup>+/-</sup>, and *Vegfb*<sup>-/-</sup> mice (see Table online, <http://www.circresaha.org>). Global normothermic ischemia completely abolished contractile function in all hearts within 2 to 3 minutes and caused a rapid rise in diastolic pressure. Time to onset of contracture and peak-developed contracture are indicators of the severity of ischemic injury. Although no difference was found in the rate of contracture development, peak contracture during ischemia was greater in *Vegfb*<sup>-/-</sup> compared with *Vegfb*<sup>+/+</sup> and *Vegfb*<sup>+/-</sup> hearts (Figure 5a). Diastolic pressure was significantly elevated in *Vegfb*<sup>-/-</sup> hearts compared with *Vegfb*<sup>+/+</sup> and *Vegfb*<sup>+/-</sup> hearts during reperfusion and recovered minimally ([almost equal to]73 mm Hg) relative to the other two groups ([almost equal to]35 mm Hg) (Figure 5a). Recovery of contractile function was slightly depressed throughout reperfusion in *Vegfb*<sup>-/-</sup> hearts, with the rate-pressure product being significantly lower at 30 minutes compared with *Vegfb*<sup>+/+</sup> and *Vegfb*<sup>+/-</sup> hearts (Figure 5b). Coronary flow responses did not differ between the three groups at any time point (Figure 5c).

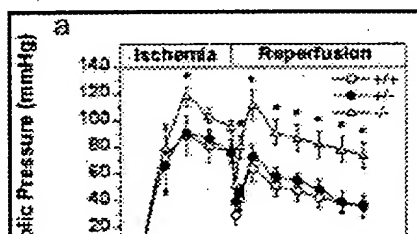
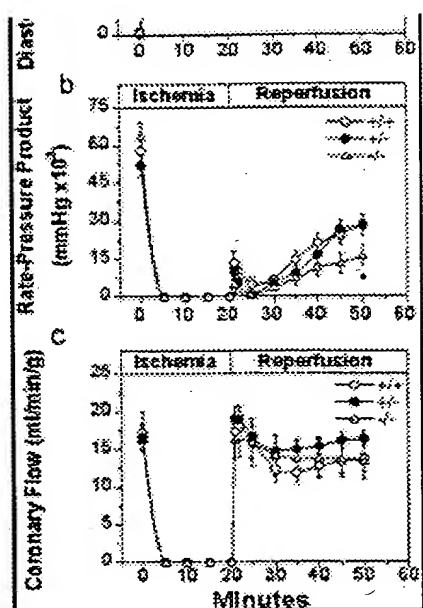


Figure 5. Functional responses of Langendorff-perfused *Vegfb*<sup>+/+</sup>, *Vegfb*<sup>+/-</sup>, and *Vegfb*<sup>-/-</sup> hearts to 20 minutes of global normothermic ischemia and 30 minutes of reperfusion. a, Responses for left ventricular diastolic pressure. Rate-pressure product (heart rate×left ventricular developed pressure) (b) and coronary flow (c) are shown. Values are mean±SEM. \**P* < 0.05 vs *Vegfb*<sup>+/+</sup> hearts.



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## Discussion

In the present study, we have used promoter trap *LacZ* expression in the *Vegfb*<sup>+/+</sup> mouse to correlate Vegf-B/[beta]-Gal expression to processes of vascularization in the heart, the major site of Vegf-B expression. The general developmental pattern of Vegf-B expression has previously been interpreted to reflect a paracrine action of Vegf-B on the developing vasculature, although no attempts were made to correlate expression patterns with developmental processes of vascularization in any individual organ. <sup>16</sup> Although *Vegfb* transcripts are produced in the heart from E8.5, Vegf-B protein expression is not detected until E10.5. <sup>16</sup> We can first detect Vegf-B/[beta]-Gal at E10.5 in the interventricular septum and propose that Vegf-B/[beta]-Gal (and therefore Vegf-B) production increases substantially at this time point, in spatial and temporal correlation with the commencement of coronary endothelial growth in the heart. <sup>23</sup> Indeed, Vegf-B/[beta]-Gal levels increase both throughout development and after birth, closely correlating with the progression of cardiac angiogenesis. Vegf-B/[beta]-Gal is conspicuous in the subepicardium where heart angiogenesis has been shown to commence. <sup>24</sup> It is more densely expressed in the ventricles than the atria and correlates with the degree of coronary angiogenesis that is more advanced in the ventricles than in the atria at fetal stages. After birth, Vegf-B/[beta]-Gal expression increases further in the LV, at a time when substantial capillary growth occurs predominantly in this chamber. <sup>21,22</sup> The disparity between LV and RV capillary growth rates decreases several days after birth. <sup>21</sup> Accordingly, by P25, Vegf-B/[beta]-Gal expression becomes even throughout the heart with levels of expression similar in RV, LV, and the atria. Despite the correlation between capillarization and Vegf-B/[beta]-Gal expression, such expression was not found in the smooth muscle cells of the differentiated coronary vessels. It is therefore likely that Vegf-B may exert its paracrine action on the microvasculature surrounding the expressing myocardium, rather than on the endothelium of the coronary vessels.

We find that *Vegfb*<sup>-/-</sup> hearts appear morphologically and functionally normal in the unstressed animal but do not undergo the same extent of postnatal growth as those of *Vegfb*<sup>+/+</sup> and *Vegfb*<sup>+/+</sup> animals. Postnatal heart growth appears to be mainly due to the substantial increase in the coronary microvasculature and vessels. <sup>11,22</sup> This increase has been attributed to the action of Vegf-A<sub>164</sub> and Vegf-A<sub>188</sub>, <sup>11</sup> because mice lacking these Vegf-A isoforms die as a consequence of severe heart ischemia due to an almost total absence of postnatal capillary and coronary vessel growth. Postnatal ablation of Vegf-A (and possibly Vegf-B) function by administering a soluble Flt-1 receptor (mFlt(1-3)-IgG) <sup>25</sup> is also lethal. In the heart, this treatment leads to cardiomyocyte necrosis and massive capillary and vessel density reduction. <sup>25</sup> Because Vegf-B is coexpressed with Vegf-A in the myocardium of the ventricles, <sup>6</sup> can form biologically active heterodimers with Vegf-A, and also binds Flt-1, <sup>7</sup> it is likely that the abnormal coronary angiogenesis described above is a result of interference

with the normal function of both Vegf-A and Vegf-B. We tested whether the observed *Vegfb*<sup>-/-</sup> reduction in heart weight was a consequence of impaired growth of the vascular network by measures of coronary capillary and vessel density. We found no significant differences between *Vegfb*<sup>-/-</sup> and *Vegfb*<sup>+/-</sup> hearts, although additional studies measuring lumen size, patency, and permeability of capillaries and vessels in the heart will reveal whether any structural abnormalities in the vascular network of *Vegfb*<sup>-/-</sup> hearts may be responsible for reduced volume of this organ. Alternatively, the observed microcardia could be attributed to an effect of Vegf-B on cardiomyocyte growth. Vegf-B effect on heart muscle could be mediated by the Vegf-B<sub>167</sub> (and Vegf-A<sub>165</sub>) receptor, neuropilin-1, which is expressed in the developing cardiac muscle. However, this is unlikely, because cardiomyocytes do not appear affected in size or function in the *Vegfb*<sup>-/-</sup> heart, and we cannot rule out a direct effect of Vegf-B ablation on myocytes. It is worth noting, nevertheless, that cardiomyocytes are normal in the *Vegfa*<sup>120/120</sup> mouse, where neuropilin-1 ligand Vegf-A<sub>165</sub> has been ablated. <sup>11</sup> A slight decrease in left ventricular thickness in the *Vegfb*<sup>-/-</sup> heart may indicate that some developmental hypoplasia, resulting from suboptimal vascularization, could be responsible for the observed microcardia.

Ablating *Vegfb* expression reduced the ability to repay coronary flow after a transient coronary occlusion. This occurred despite baseline coronary flow in the *Vegfb*<sup>-/-</sup> heart appearing normal, which was not unexpected given that moderate impairment of vascularization or vascular function that might result from deletion of the *Vegfb* gene could be compensated by enhanced intrinsic vasodilatation. Impairment of flow-debt repayment, despite similar peak flows, suggests inhibition of flow-mediated dilatation, which occurs subsequent to the immediate hyperemic response, indicating that the functional status of the coronary vasculature is impaired in some way in *Vegfb*<sup>-/-</sup> hearts. Reactive hyperemia is thought to be mediated by the combined actions of nitric oxide (NO) and adenosine, <sup>26</sup> with potential involvement of K<sub>ATP</sub> channels. <sup>27</sup> The prolongation of the hyperemic response is thought to be at least partially NO dependent. <sup>28</sup> Thus, one possible mechanism contributing to this change is an impaired NO production. However, deletion of the endothelial NO synthase gene fails to alter peak hyperemic flow, flow repayment, and adenosine responses in murine hearts. <sup>29</sup>

Heart rate was almost identical in hearts of all genotypes before and after ischemia, and no significant differences existed for heart rate between any groups at any time. Interestingly, deletion of *Vegfb* reduced functional recovery from ischemia-reperfusion and appeared to worsen contracture during ischemia. The mechanism of contracture is not well understood but may involve rigor-bond formation as a result of impaired glycolytic ATP formation. <sup>30</sup> During reperfusion, diastolic dysfunction was significantly greater in knockout mice; the difference was wholly due to a change in contractile force and not rate. Recovery of the rate-pressure product was slightly reduced whereas coronary flow was similar in all three groups. Although a reduced reflow or perfusion could have explained the dysfunction, this was not supported by the measures of total myocardial perfusion. However, this does not exclude a more subtle change in flow distribution that is not reflected in the total flow response. The postischemic elevation in diastolic pressure is likely to reflect altered Ca<sup>2+</sup> handling in reperfused tissue, resulting in enhanced diastolic Ca<sup>2+</sup> levels. <sup>31</sup> Ca<sup>2+</sup> handling is energy dependent, particularly at the level of the sarcoplasmic reticulum. Knockout of the *Vegfb* gene could conceivably lead to impaired postischemic recovery of energy metabolism, owing to maldistribution of coronary flow, such that myocardial handling of Ca<sup>2+</sup> is impaired. Further experiments addressing patency, permeability, and responses to vasodilatory stimuli in the ventricular microvasculature of *Vegfb*<sup>-/-</sup> hearts will reveal whether this is indeed the case. The increased diastolic dysfunction during ischemia is largely independent of the coronary vasculature and may reflect a developmental effect of *Vegfb* deletion on heart growth or function, as suggested by the smaller hearts and reduced left ventricular thickness in *Vegfb*<sup>-/-</sup> mice.

In the present study, we have shown that, despite heart morphology and function being normal in *Vegfb*<sup>-/-</sup> mice, the response to coronary occlusion and myocardial recovery from ischemia are compromised. Thus, although Vegf-B may play a redundant role in establishing the coronary vasculature, our results define a unique role in the development and maintenance of function in response to ischemic insult.

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Key Words: angiogenesis; cardiac ischemia; coronary vasculature



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COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

IN THE MATTER OF: Australian  
Patent Application 696764  
(73941/94). In the name of:  
Human Genome Sciences Inc.

- and -

IN THE MATTER OF: Opposition  
thereto by Ludwig Institute for Cancer  
Research, under Section 59 of the  
Patents Act.

Annexure GBC-5

This is **Annexure GBC-5** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
\_\_\_\_\_  
Gary Baxter Cox

WITNESS:

  
\_\_\_\_\_  
Patent Attorney

PEYTEE KINE

# STRATAGENE

CLONING SYSTEMS

1994

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*Creating the tools for the creative mind*

## DESCRIPTION

- Direct secretion of recombinant proteins into insect cell supernatant
- Simplifies purification and characterization of expressed proteins
- Ease of purification further enhanced by serum-free medium
- Choice of 2 different signal sequences
- Fusion genes transcribed under strong polyhedrin promoter
- Compatible with all AcNPV expression systems

## UNIQUE SECRETORY SIGNAL SEQUENCES

pMbac and pPbac contain secretory signal sequences that direct the nascent polypeptide chain toward the secretory pathway of the cell, thereby leading to secretion into the cell supernatant. The sequences to be expressed are inserted 3' to the signal sequences to generate a fusion gene that is transcribed under the strong polyhedrin promoter. The signal sequence is cleaved off by signal-sequence peptidase as the nascent polypeptide is channeled toward the secretory pathway of the host insect cell, leading to the secretion of mature recombinant protein. Proper processing of signal peptidase at the expected cleavage site has been verified by protein sequencing of secreted material.

## EASY PURIFICATION OF RECOMBINANT PROTEINS

With no need for cell lysis, purification of the secreted recombinant proteins is extremely easy. The process starts with simple separation of the insect cells from the supernatant. Downstream processing of the protein is made even more efficient when the pMbac and pPbac transfer vectors are used with Stratagene's Cell/Perfect™ Bac serum-free insect cell culture medium.

## PROTOCOL FOR USE WITH BACULOVIRUS EXPRESSION SYSTEMS

The pMbac and pPbac baculovirus transfer vectors are compatible with systems based on the baculovirus species most commonly used for expression work, *Autographica californica* nuclear polyhedrosis virus (AcNPV). Once in the nucleus of infected cells, the baculovirus expresses the protein polyhedrin late in the infectious cycle under a very strong promoter. The polyhedrin gene is not essential for viral propagation in tissue culture, so it can be replaced by cDNA whose expression will then be driven by the strong polyhedrin promoter.

To replace the polyhedrin gene in the baculovirus genome:

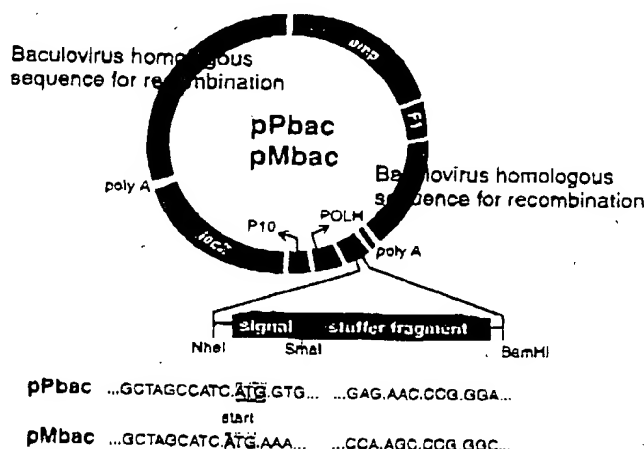
1. Insert the cDNA fragment into the pMbac or pPbac transfer vector, each of which contains recombination sequences.
2. Cotransfect the baculovirus DNA and transfer vector into the insect cell line SF9. The resulting double recombination event replaces the polyhedrin gene with the vector fragment containing the gene to be expressed.
3. Identify recombinant virus based on the presence of the *lacZ* gene in the recombination fragment.

## CONTENTS

- 20 µg of pMbac and/or pPbac transfer vector
- 2.5 µg each of forward and reverse sequencing primers

## REFERENCES

1. Lemhardt, W., et al. (1993) *Strategies* 6: 20-21.



## Baculovirus Transfer Vectors

pMbac and pPbac

Catalog # 211502

S225

pMbac

Catalog # 211503

S125

pPbac

Catalog # 211504

S125

## Baculovirus Sequencing Primers

Forward primer

2.5 µg

Catalog # 300313

S105

Reverse primer

2.5 µg

Catalog # 300314

S105

## Cell/Perfect™ Bac Serum-Free Insect Cell Culture Medium

1 liter

Catalog # 205120

S35

## Map of Baculovirus Transfer Vectors

To create the pMbac and pPbac vectors, the melittin and human placental alkaline phosphatase secretory signal sequences were introduced into the *Nhe*I and *Bam*HI sites, respectively, of the transfer vector pJVP10Z. Therefore, genes to be expressed can be introduced unidirectionally into the *Sma*I/*Bam*HI sites of the vectors.



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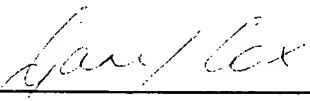
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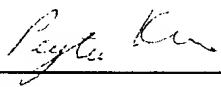
- and -

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thereto by Ludwig Institute for Cancer  
Research, under Section 59 of the  
Patents Act.

Annexure GBC-6

This is **Annexure GBC-6** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
\_\_\_\_\_  
Gary Baxter Cox

WITNESS:   
\_\_\_\_\_  
Patent Attorney      PEYTEE K. LOO

# Molecular and Cell Biology Catalog 1992



## Vector Applications Guide (cont.)

Pr motor	Induction	ATG	RBS	Translation termination	Enzymatic cleavage	Prokaryotic Gene Fusion Vectors
pGEX-1 $\lambda$ T						<ul style="list-style-type: none"><li>• Induction: <i>tac</i> promoter inducible with 1–5 mM IPTG.</li><li>• Expression: Proteins are expressed as fusion proteins with the 26-kDa glutathione S-transferase (GST). The GST gene contains an ATG and ribosome-binding site, and is under control of the <i>tac</i> promoter. Translation terminators are provided in all three reading frames. The resulting fusion protein may be purified on Glutathione Sepharose (17-0756-01) or on prepacked Glutathione Sepharose columns (17-0757-01).</li><li>• Enzymatic cleavage: For pGEX-1<math>\lambda</math>T: The GST carrier protein may be separated from the fusion protein using the site-specific protease thrombin. For pGEX-2T: The GST carrier protein may be separated from the fusion protein using the site-specific protease thrombin. For pGEX-3X: The GST carrier protein may be separated from the fusion protein using the site-specific protease factor Xa.</li><li>• Reading frame: For pGEX-1<math>\lambda</math>T: The reading frame at the <i>EcoR</i> I site is GAA TTC ATC. This is compatible with the <i>EcoR</i> I site in <math>\lambda</math>gt11. For pGEX-2T: The reading frame at the <i>EcoR</i> I site is GGA ATT CAT. For pGEX-3X: The reading frame at the <i>EcoR</i> I site is GGG AAT TCA.</li><li>• Host(s): <i>E. coli</i>. The plasmid provides <i>lac</i><sup>I</sup> repressor.</li><li>• Selectable marker(s): Plasmid confers resistance to 50 <math>\mu</math>g/ml ampicillin.</li><li>• Amplification: Recommended.</li></ul>
<i>tac</i>	X	X	X	X	X	
pGEX-2T						
<i>tac</i>	X	X	X	X	X	
pGEX-3X						
<i>tac</i>	X	X	X	X	X	
pRIT2T						<ul style="list-style-type: none"><li>• Induction: The lambda P<sub>R</sub> promoter is induced by shifting the growth temperature from 30°C to 42°C for 90 minutes.</li><li>• Expression: Proteins are expressed as fusion proteins with <i>Staphylococcal</i> protein A. The lambda <i>cro</i> gene supplies an ATG. No signal sequence is provided; therefore the protein remains intracellular. Transcription and translation termination signals are provided. Fusion protein may be purified on IgG Sepharose (17-0969-01). The size of the protein A carrier protein is about 24 kDa.</li><li>• Host(s): N4830-1/NC991*. N4830-1 must be used for expression, as it contains the temperature-sensitive <i>cI857</i> repressor.</li><li>• Selectable marker(s): Plasmid confers resistance to 100 <math>\mu</math>g/ml ampicillin.</li></ul>
$\lambda$ P <sub>R</sub>	X	X	X	X		
pEZZ 18						<ul style="list-style-type: none"><li>• Expression: Expression is controlled by both the <i>lacUVS</i> and protein A promoters and is not inducible. Proteins are expressed as fusions with the synthetic ZZ peptide which is based on an IgG binding domain of protein A. The protein A signal sequence is provided so expression in <i>E. coli</i> leads to secretion of fusion proteins into the culture medium. Elements of the protein A gene provide both the ATG and ribosome-binding site. Stop codons must be provided by the insert. Fusion protein may be purified on IgG Sepharose (17-0969-01). The size of the ZZ carrier is about 14 kDa.</li><li>• Sequencing: M13 Universal Sequencing Primer and M13 -40 Sequencing Primer can be used for both double-stranded and single-stranded sequencing. A protocol for production of single-stranded DNA is provided with the vector.</li><li>• Cloning: Inserts containing a stop codon will yield white colonies when grown on media containing X-gal.</li><li>• Host(s): <i>E. coli</i>.</li><li>• Selectable marker(s): Plasmid confers resistance to 70 <math>\mu</math>g/ml ampicillin.</li><li>• Amplification: Recommended.</li></ul>
<i>spal/UVS</i>		X	X			
pMC1871						<ul style="list-style-type: none"><li>• Expression: The <i>lac</i> gene is promoterless and missing the first eight non-essential amino acids. Inserts cloned into the <i>Sma</i> I site give fusion proteins with <math>\beta</math>-gal. Insert must contain a promoter, ATG, and ribosome-binding site.</li><li>• Host(s): <i>E. coli</i> strains carrying a <i>lac</i> deletion.</li><li>• Selectable marker(s): Plasmid confers resistance to 15 <math>\mu</math>g/ml tetracycline.</li></ul>
			X			

Note: For high-level transformation of host cells (*E. coli*), we recommend the "Hanahan" protocol [Hanahan, D., *J. Mol. Biol.* 166, 557 (1983)].

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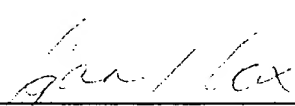
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- and -

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thereto by Ludwig Institute for Cancer  
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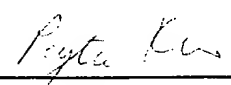
Annexure GBC-7

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Thirteenth day of December 2000.

  
\_\_\_\_\_  
**Gary Baxter Cox**

WITNESS: \_\_\_\_\_

Patent Attorney

  
PETEE L. HALL

## Corrigendum/Erratum

### A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases

Vladimir Joukov, Katri Pajusola, Arja Kaipainen, Dmitri Chilov, Isto Lahtinen, Eola Kukk, Olli Saksela, Nisse Kalkkinen and Kari Alitalo

*The EMBO Journal*, 15, 290–298, 1996

Our recent results on the nascent VEGF-C polypeptide immunoprecipitated from metabolically labelled cells suggest that the intracellular precursor protein is larger than the secreted protein, for which we predicted the open reading frame given in our paper in *EMBO J.*, 15 (2), 290–298 (1996). This would suggest that an upstream ATG codon at position 352 in the same reading frame of the VEGF-C cDNA sequence (accession number X94216) is used for initiation of translation, resulting in an additional N-terminal 'prepro-VEGF-C' peptide: MHLLGFFS-VACSLAALLPGPREAPAAAAAFESGLDLSDAEP-DAGEATAYASKDLEEQLRSVSSVDEL. This sequence is identical to the N-terminus of the peptide sequence of Lee *et al.* (U43142), submitted to the database on December 12, 1995. The numbering of amino acid residues of VEGF-C presented in Figure 3B would then be 70–419 and in Figure 3C 275–365 (starting from the first methionine residue of the 'prepro-VEGF-C'). The sequence underlined in Figure 3B thus represents the C-terminal part of the 'prepro-VEGF-C' peptide rather than the signal peptide. However, the alignment of the homologous sequences in Figure 3B and C remains unchanged. The predicted molecular mass of the entire VEGF-C precursor is 46 883 and the length is 419 amino acid residues. Our EMBL, GenBank and DDBJ entry X94216 includes these features.

The sentence on the title page 290: 'B.Olofsson and K.Pajusola contributed equally to this work, as did K.Alitalo and U.Eriksson' was erroneously copied by Oxford University Press from the given title page of the reference: Olofsson, B. *et al.* (1996) *Proc. Natl Acad. Sci. USA*, in press.

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Patent Application 696764  
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Annexure GBC-8

This is **Annexure GBC-8** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
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**Gary Baxter Cox**

WITNESS:

  
\_\_\_\_\_  
Patent Attorney

PEYTEE K. HOO

# Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (*flt*) closely related to the *fms* family

Masabumi Shibuya, Sachiko Yamaguchi, Akira Yamane, Toshio Ikeda, Arinobu Tojo, Hitoshi Matsushime & Misako Sato

Department of Genetics, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108, Japan

A new human gene encoding a receptor-type tyrosine kinase was isolated by a weak cross-hybridization with *v-ras* oncogene. A cDNA of about 7.7 kb carried a 4.2 kb open reading frame, and the predicted amino acid sequence of 1338 residues contained extracellular, transmembrane and tyrosine kinase domains. Although its extracellular domain is approximately 220 amino acids longer than those of the products of the *fms* family, i.e. *c-fms*, *c-kit* and platelet-derived growth factor receptor genes, the overall structure including cysteine motifs in its extracellular domain and a long peptide insertion in its tyrosine kinase domain indicates that this new gene is closely related to the *fms* family. Consequently, the gene was designated as *flt* (*fms*-like tyrosine kinase) gene. The expression of the *flt* gene was strongly suppressed in most of the tumor cell lines examined so far, whereas this mRNA was expressed in a variety of normal tissues of adult rat.

## Introduction

Most of the tyrosine kinase genes carry potential transforming activities, and appear to have important roles in the signal transduction pathways for cell growth and differentiation in normal conditions (Bishop, 1985). Up to now, more than 30 genes of tyrosine kinase have been molecularly cloned. However, the large number of peptide hormones and factors reported in animal tissues suggest that many important receptor genes still remain to be isolated from a variety of tissues in higher organisms.

Tyrosine kinase genes can be divided into two major groups: one a group of non-receptor-type genes such as *src* of Rous sarcoma virus and the other a group of receptor-type genes. Similar to the case of non-receptor-type tyrosine kinase genes, receptor-type tyrosine kinase genes contain several gene families with respect to nucleotide and amino acid homology: (1) EGF receptor gene (*c-erbB*)/*c-erbB2*(*neu*) genes, (2) insulin-receptor/insulin-like growth factor I receptor genes, and (3) the *c-fms* gene families. The *c-fms* family includes three biologically unique genes, colony stimulating factor-1 (CSF-1) receptor gene (*c-fms*) (Hampe *et al.*, 1984; Sherr *et al.*, 1985), platelet-derived growth factor (PDGF) receptor gene (Yarden *et al.*, 1986; Matsui *et al.*, 1989) and *c-kit* gene which is the cellular counterpart of *v-kit* gene obtained from HZ4 feline sarcoma virus (Besmer *et al.*, 1986; Yarden *et al.*, 1987; Qiu *et al.*, 1988). The characteristics of this family are a long peptide insertion in the middle of the tyrosine kinase domain and a

unique distribution of cysteine residues in the extracellular domain. Furthermore, the ligands identified for this family, CSF-1 and PDGF, consist of a dimer structure with subunits of approximately 20000 daltons connected with disulfide bonds (Waterfield *et al.*, 1983; Johnsson *et al.*, 1984; Stanley & Heard, 1977; Das & Stanley, 1982). Although the ligand and the physiological functions of the *c-kit* gene are not yet clear, recent studies revealed that this gene is tightly linked to the murine White (W) locus which has pleiotropic effects on embryonic development and hematopoiesis (Chabot *et al.*, 1988; Geissler *et al.*, 1988).

Recently we have molecularly cloned a human genomic DNA fragment bearing an exon for a portion of a new tyrosine kinase gene, probed with *v-ras* sequence under a hybridization condition of low stringency (Matsushime *et al.*, 1987). An *in situ* hybridization analysis revealed that this new gene (*flt*) is located on human chromosome 13q12-13 where no protein kinase gene has so far been assigned (Sato *et al.*, 1987). As an initial step to elucidate the physiological significance of *flt* gene in normal tissues and its potential transforming activity, we isolated *flt* cDNA clones and determined its primary structure. Here we report the entire coding sequence and the predicted protein structure of the *flt* gene, which is found to be a new member of the *fms* family.

## Results and discussion

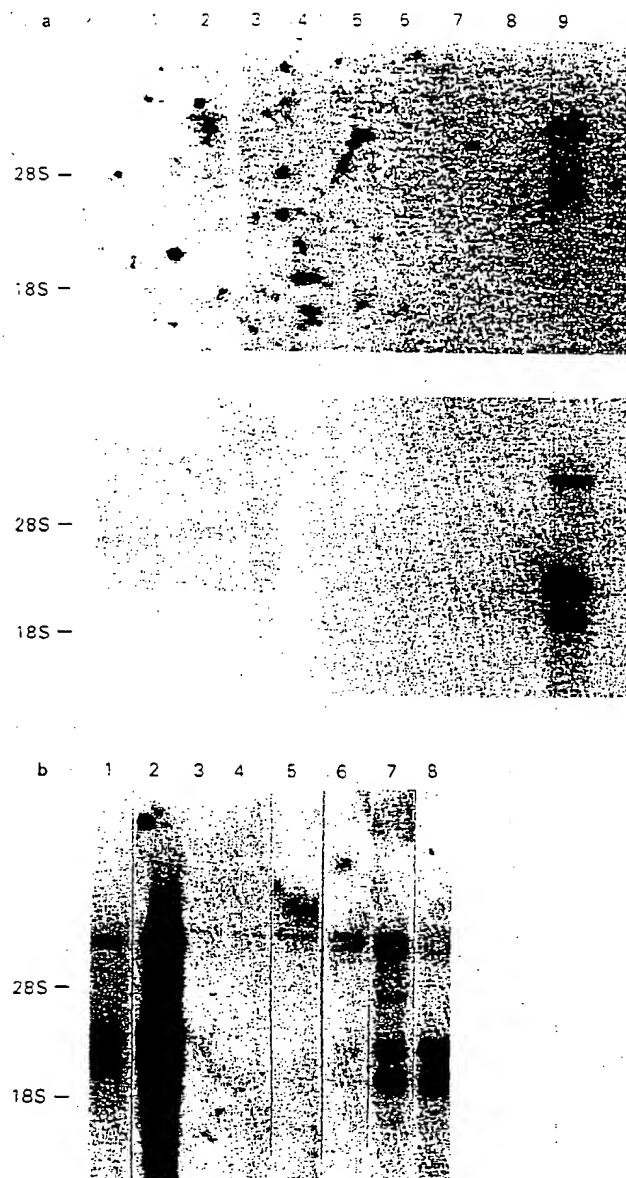
### Isolation and nucleotide sequencing of *flt* cDNAs

We previously obtained from a human genomic library a DNA fragment (*flt*) which contains an exon 123 base pairs (bp) long (Matsushime *et al.*, 1987). Using this DNA as a probe, expression of the *flt* gene was surveyed in several human cell lines and placenta tissue by Northern blot analysis (Figure 1a). We could detect a faint band of approximately 8.0 kb both in placenta and in a cell line 293.

To isolate the relevant cDNA molecule, we constructed a human placenta cDNA library which contains relatively long cDNA molecules (see Materials and methods). Among 7 *flt* cDNA clones obtained, two overlapping clones, #3-7 (5.6 kb) for the 5'-half region and #3-5 (4.5 kb) for the 3'-half region were sequenced by the dideoxynucleotide method (Sanger *et al.*, 1977).

### The predicted structure of the *flt* gene product

The entire cDNA sequence of 7680 bp is shown in Figure 2. A long open reading frame starts at the nucleotide residue 1 and continues for about 4.2 kb. The first ATG codon exists at the nucleotide residue 250-252. Although no termination codons are present



**Figure 1** Northern blot analysis of the human *fit* gene. Poly (A)<sup>+</sup> RNA was prepared from various tissues and cell lines (see Materials and methods), and 2 µg of these RNAs were separated on a gel, transferred to a nitrocellulose filter and hybridized with EcoRI-BamHI 0.8 kb fragment of human genomic *fit* DNA (Matsushima *et al.*, 1987) (upper panel in a) or with #3-7 *fit* cDNA (lower panel in a, and b) as a probe. RNA samples in (a): lane 1, KB cell; lane 2, 293 cell (a cell line derived from human embryo kidney); lane 3, Daudi (B-lymphoma); lane 4, Namalwa (B-lymphoma); lane 5, Kato-III (human gastric adenocarcinoma cell line); lane 6, Ito-II (human testicular tumor cell line); lane 7, Molt-4 (T-lymphoma); lane 8, B16 (mouse melanoma cell line); lane 9, human placenta. RNA samples in (b): lane 1, human placenta (low amount of RNA, 0.2 µg); lane 2, human placenta; lane 3, human liver; lane 4, human muscle; lane 5, human kidney; lane 6, 293 cell line; lane 7, 293E1 cell line; lane 8, BeWo (human choriocarcinoma cell line)

upstream from this ATG codon, we consider that this ATG is the initiation codon of *fit* gene product for the following reasons: (1) the nucleotide residue 1 to 249 are extremely GC-rich (approximately 80%) and this characteristic is similar to that of the upstream noncoding

region of several other receptor-type tyrosine kinase genes, i.e. EGF receptor and insulin receptor genes (Ullrich *et al.*, 1984; Ebina *et al.*, 1985; Ullrich *et al.*, 1985), (2) a short stretch including the 250-252 ATG codon, TCACCATGG, is well matched with Kozak's criteria CC(A/G)CCATGG for the initiation codon in mammalian species (Kozak, 1984); (3) this ATG is followed by 21 codons which are mostly for hydrophobic amino acids, and therefore the features of this region are consistent with those of a signal peptide of membrane proteins; (4) the position of this ATG codon is identical to the position of the initiation codons in the *fms* gene family when the cysteine residues in the *fit* extracellular domain are aligned to those of the *fms* gene product. Calculating from this ATG codon as the amino acid residue 1, the predicted *fit* gene product consists of 1338 amino acid residues and the molecular weight was expected to be 150 565 daltons.

#### Possible domain structure of the *fit* gene product

The *fit* product can be subdivided into three regions: a 758-amino-acid extracellular domain; a 22-amino-acid transmembrane domain which is followed by a cluster of basic amino acids (Arg-Lys-Met-Lys-Arg); a 558-amino-acid cytoplasmic region containing a tyrosine kinase domain. This kinase domain has a Gly-x-Gly-x-x-Gly stretch at the residues 834-839, a conserved lysine at the ATP binding site (residue 861) and a tyrosine residue (#1053) as the putative autophosphorylation site which corresponds to tyrosine-#416 in the *src* gene product. One of the most remarkable features of the *fit* tyrosine kinase domain is a very long peptide (66 amino acids) insertion at the middle of the kinase domain. The position and the length of this insert are essentially the same as those in the *fms* gene family (Figure 3). Furthermore, a striking similarity between *fit* gene product and the *c-fms* gene family was also detected in the distribution of cysteine residues within the extracellular domain, as shown in Figures 3 and 4. Thus, the *fit* gene appears to belong to the *fms* family, and also to the 'Immunoglobulin superfamily' in which cysteine residues form intramolecular disulfide bonds for appropriate folding of ligand binding domain (Williams, 1989).

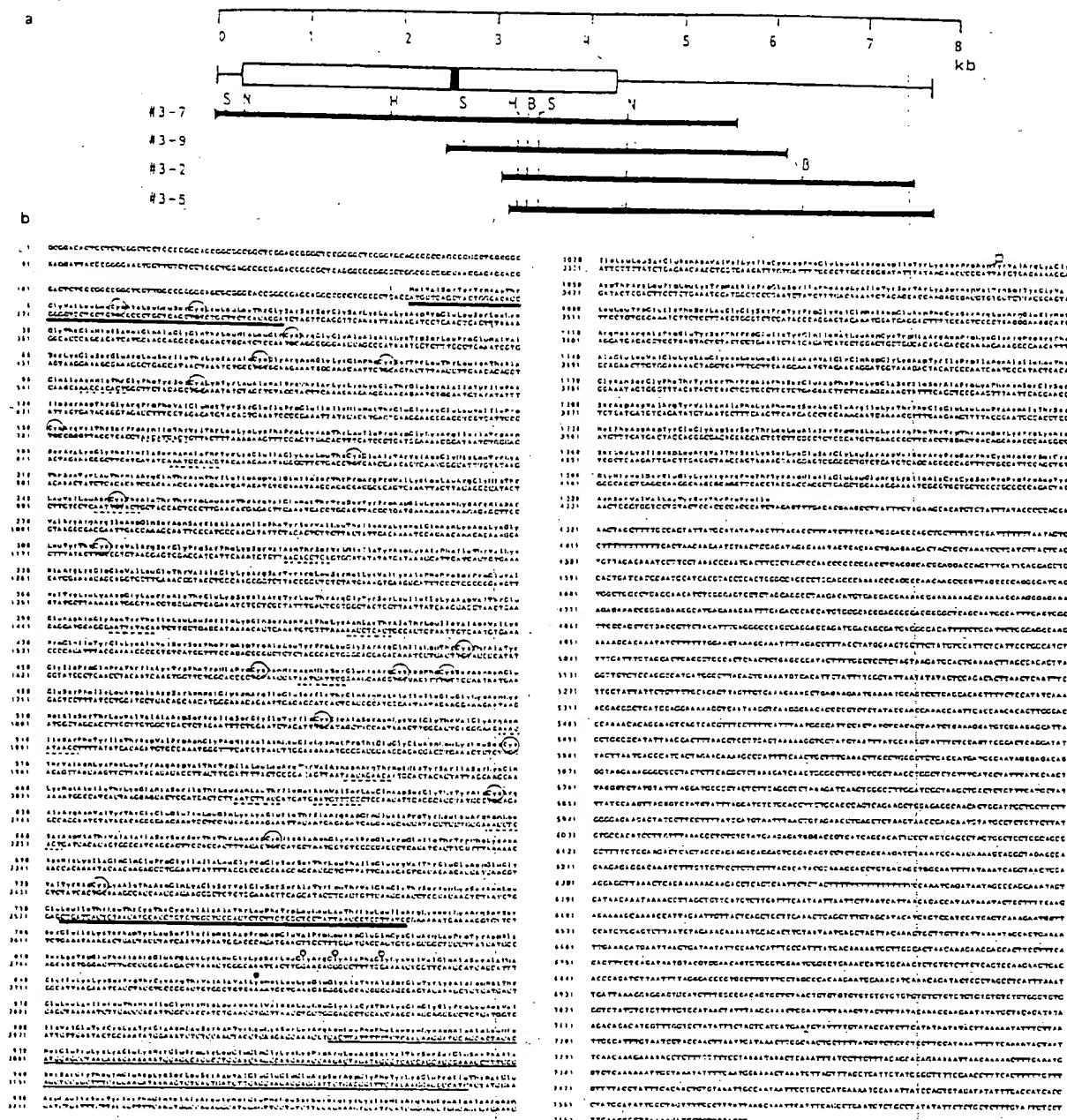
However, a clear structural difference exists between the *fit* gene product and the *fms* family: the length of the extracellular domain in the *fit* gene product is about 220 amino acids longer than those in the *fms*, *kit* and PDGF receptor gene products. Although there are several possible explanations for the origin of this *fit*-specific region (Stretch *et al.* in Figure 4), a partial gene duplication in the extracellular domain seems most likely, because a weak but significant amino acid homology was observed between the *fit*-specific sequence (residues 550-745) and the 230-amino-acid region just upstream of the transmembrane domain in the *fms* family (Figure 4). Recently we have confirmed the presence of this *fit*-specific region in murine *fit* cDNA (Yamane & Shibuya, unpublished results).

#### Homology between *fit* and other tyrosine kinase genes

Since the *fit* gene was originally isolated on the basis of a weak nucleotide homology with *v-ros* DNA, the overall homologies of the *fit* tyrosine kinase domain except for the insert region at the amino acid level were

col  
531  
fm  
tyr  
kir  
src  
clc  
kir  
far





**Figure 2** Nucleotide sequence and predicted amino acid sequence of the *flt* cDNA. (a) Representative cDNA clones, restriction sites and the open reading frame are indicated. (b) Nucleotide and amino acid sequences in the *flt* gene. The number of amino acid residues is started from the putative initiation codon at nucleotide residue 250-252. A heavy underline at the amino terminal region indicates a possible signal peptide. Another heavy underline in the middle of the sequence shows putative transmembrane domain. Cysteine residues within the extracellular domain (1-19) are encircled. Potential N-glycosylation sites in the same domain are shown by broken lines. Small open circles indicate glycine residues at G-X-G-X-X-G region in the tyrosine kinase domain, and closed circle represents putative ATP binding site. Thin underline in the middle of the cytoplasmic domain indicates the long insert within the kinase domain, and the open square shows the tyrosine residue for a possible autophosphorylation. B, BglII; H, HindIII; N, NcoI; S, SmaI

compared among various other protein kinase genes. As shown in Table 1, the homology was higher with the *fms* family (54-60%), whereas those with other receptor-type (including *c-ros* gene) or non-receptor-type tyrosine kinase genes were lower (35-38%). Thus the homology score further supports the hypothesis that the *flt* gene is closely related to the *fms* family (Figure 5).

Although the amino acid homology in the tyrosine kinase domain is remarkable between the *flt* and *fms* families, the long peptide insert in the middle of the *flt*

tyrosine kinase domain has little or no significant homology with the insert of any member of the *fms* family. Thus, this region might have a role in specific function(s) of each gene product of the *c-fms/flt* family. Recently, Escobedo & Williams (1988) have reported that a mutant of PDGF receptor carrying a deletion within the insert region in the kinase domain cannot stimulate mitogenic response in spite of exhibiting tyrosine kinase activity and other biological functions in response to PDGF.

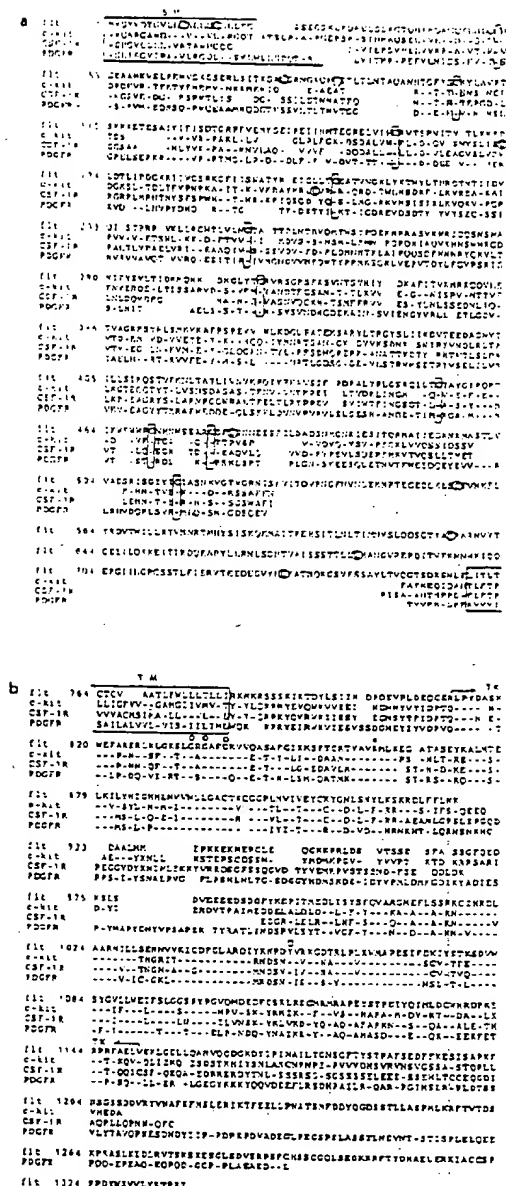


Figure 3 Comparison of the amino acid sequences between *fit* gene and other members of *fms* family (*fms*, *kit*, and PDGF receptor). The amino acid sequences were aligned and gaps (empty space) were introduced for optimal homology. Amino acids in the *fms* family identical to those in the *fit* gene product are indicated by a dash. The sequence of PDGF receptor is that reported by Yarden *et al.* (1986). (a) Similarity of the extracellular domain. The regions from signal peptides (SP) to the middle of transmembrane domains are indicated. All cysteine residues are circled or boxed. Amino acid residues #550 to #745 are *fit*-specific. (b) Similarity of the intracellular domain. The regions from the middle of transmembrane domain (TM) to the carboxyl ends are indicated. Three conserved glycine residues, the lysine for the potential ATP binding site and the potential tyrosine autophosphorylation site are indicated by open circles, by a closed circle and by an open square, respectively. The region of tyrosine kinase domain is shown by arrows.

#### Expression of the *fit* gene in cell lines and normal tissues

In order to study the physiological significance of the *fit* gene, its expression was examined by Northern blot analysis using molecularly cloned *fit* cDNA. Poly(A)<sup>+</sup> RNAs obtained from a variety of normal tissues and

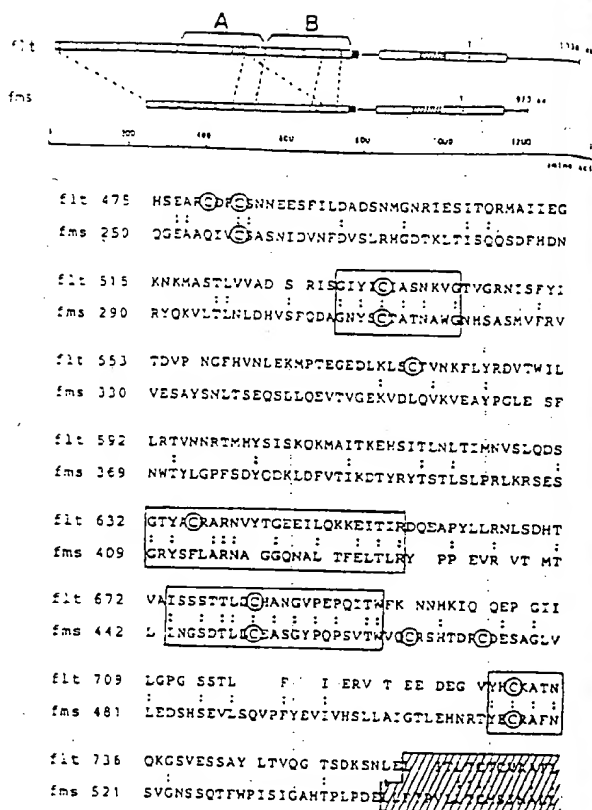


Figure 4 A possible partial gene duplication of extracellular domain in the *fit* sequence. About 280 amino acid residues upstream from the transmembrane domain (shaded box) in the *fit* and the *fms* genes are compared. Highly conserved regions are boxed and all cysteine residues are circled. Sequences in (a) and in (b) are considered to be partially duplicated in the *fit* genome.

cell lines were hybridized with #3-7 5.6 kb human *fit* cDNA. As shown in Figure 1b, a higher expression was detected in human placenta tissue, and weakly hybridized bands were observed in human embryo kidney-derived cell lines, 293 and 293E1, and a choriocarcinoma cell line, BeWo (Graham *et al.*, 1977; Chinnadurai *et al.*, 1978; Pattillo *et al.*, 1968). In addition, faint bands were also detected in liver, muscle and kidney of human.

To examine further the tissue-specific expression of the *fit* gene in animals, we molecularly cloned the entire

Table 1 Amino acid homology in tyrosine kinase domain between *fit* gene and other genes of *src* family

Gene	Amino-half* %	Insert** %	Carboxyl-half† %
v- <i>fms</i>	57	5	53
PDGF-R $\alpha$ ††	59	13	58
PDGF-R $\beta$ ‡	57	13	53
v- <i>kit</i>	62	11	58
v- <i>erbB</i>	26	—	42
v- <i>ros</i>	32	—	43
v- <i>src</i>	30	—	38
v- <i>sps</i>	32	—	41

\* *fit* residue #813-#929

\*\* #930-#994

† #995-#1152

†† PDGF-R $\alpha$ : Matsui *et al.*, 1989

‡ PDGF-R $\beta$ : Yarden *et al.*, 1986

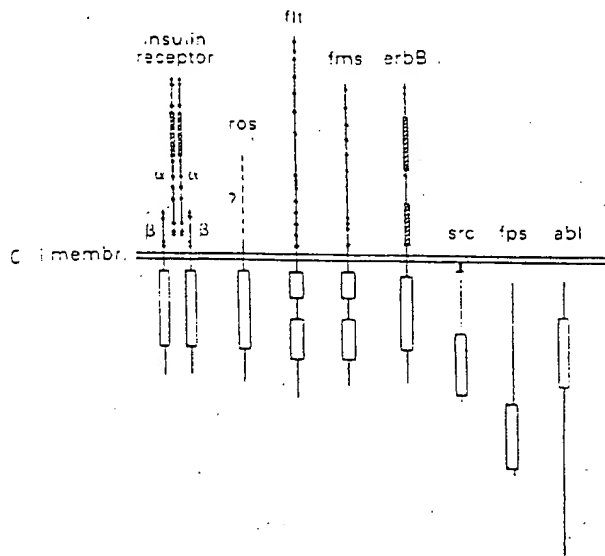


Figure 5 Schematic comparison of the *flt* gene product with those of other representative tyrosine kinase genes

coding region of *flt* cDNA of rat, and both human and rat *flt* cDNAs were used as probes. The nucleotide sequence of rat *flt* cDNA will be described elsewhere (Yamane & Shibuya, unpublished). In adult tissues of rat, *flt* mRNA was detected in many tissues such as lung, placenta, liver, kidney, heart and brain: the highest expression was observed in the lung (Figure 6, Table 2).

In contrast to these findings, the *flt* mRNA was not detectable or extremely low, if any, in most of the 20 human tumor cell lines examined. These malignant cell lines include epithelial and adenomatous carcinomas, T- and B-lymphomas, and leukemias of erythroid, granulocytic and monocytic lineages (Figure 1, Table 2). These results might suggest that the *flt* gene is not directly

involved in the process of cell proliferation, but in differentiation or maintenance of normal tissues in physiological conditions.

#### Subgenomic fragments of the *flt* mRNA

A genomic *flt* DNA corresponding to the middle portion of the kinase domain detected only 7.5–8.0 kb species of mRNA (Figure 1a), whereas, #3–7 cDNA probe, which bear the entire coding region of *flt* gene, detected other species of mRNA (2.2 and 3.0 kb) as well as 7.5–8.0 kb mRNA (Figure 1). As an explanation for these small-sized mRNAs, cross-hybridization of this cDNA probe with other tyrosine kinase genes seems unlikely, because most of the cells expressing 7.5–8.0 kb *flt* mRNA also showed 2.2 and 3.0 kb mRNAs when hybridized with #3–7 cDNA probe. Our preliminary results (Ikeda & Shibuya, unpublished results) indicate that these short mRNAs appear to be due to premature termination of the transcripts within the extracellular domain of the *flt* gene. Since these small *flt* mRNAs could encode for about amino-terminal half of the extracellular domain of this gene product, these molecules might have a regulatory role in physiological condition, through binding and absorbing the yet unidentified *flt* ligand.

#### Is the *flt* gene involved in carcinogenesis of animals?

A relationship between the *flt* gene and tumorigenicity in higher organisms was examined by Southern blot analysis. Approximately 100 human tumor cells (about 30 gastric cancers, 25 brain tumors, 10 colon cancers and other types) did not carry gene amplification or rearranged fragments of *flt* DNA at a detectable level (data not shown). Thus, we have no direct evidence to support the involvement of *flt* gene in carcinogenesis of animals. However, Walker *et al.* (1987) have recently reported overexpression of an 8.5 kb mRNA in experimental rat tracheal carcinoma which was detected by cross-hybridization with *v-fms* probe. It would be interesting to clarify whether this 8.5 kb *fms*-like gene in rats is a cognate cellular gene of the human *flt*.

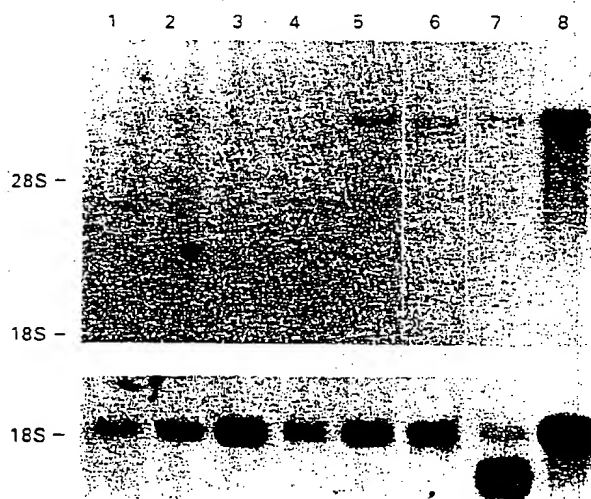


Figure 6 Expression of *flt* gene in various normal tissues of rat. About 2 µg of poly (A)<sup>+</sup> RNA was separated on an agarose gel, transferred to a nitrocellulose filter and hybridized with 0.9 kb rat *flt* cDNA corresponding to tyrosine kinase domain (upper panel) or chicken beta actin cDNA (lower panel) as probes. Lane 1, liver; lane 2, kidney; lane 3, thymus; lane 4, testis; lane 5, placenta; lane 6, brain; lane 7, heart; lane 8, lung

Table 2 Expression of *flt* gene in normal tissues and cell lines

Normal tissues		Cell line	
Human		Human	
placenta	++	BeWo (choriocarcinoma)	+
muscle	+	T3M3 (choriocarcinoma)	+
liver	+	293 (embryo kidney)	+
kidney	+	293E1 (embryo kidney)	+
Rat		Daudi (B-lymphoma)	+
placenta	+	Namalwa (B-lymphoma)	+
lung	+	Raji (B-lymphoma)	+
heart	+	Molt-4 (T-lymphoma)	+
brain	+	HL60 (promyelocytic leukemia)	+
testis	+	K562 (chronic myelogenous leukemia)	+/–
thymus	+	Hela (cervical cancer)	–
kidney	+	A431 (epitheloid cancer)	–
liver	+	Kato-III (gastric cancer)	–
		GK-T3 (small cell lung ca.)	–
		Ito-II (testicular tumor)	–
		FL (amniotic tissue)	–
		A549	–
		143B	+/–
		Mouse	
		B16 melanoma	–

## Materials and methods

## Construction of the cDNA library

Poly (A)<sup>+</sup> RNA was prepared from fresh tissue of normal human placenta and used for making a cDNA library. The library was constructed by the method described by LaPolla *et al.* (1984). Briefly, 5 µg of poly (A)<sup>+</sup> RNA was used for synthesis of the first strand cDNA with AMV reverse transcriptase and oligo(dT) primer. The second strand was then synthesized with *E. coli* DNA polymerase I and RNAase H for RNA primers. The cDNAs were treated with T4 DNA polymerase to make the ends flush, and the internal EcoRI sites were methylated. Both ends of cDNAs were ligated with EcoRI linkers and digested with EcoRI restriction endonuclease. Since the size of *flt* mRNA detected in Northern analysis was 7.5–8.0 kb, cDNAs were size-fractionated on a Bio-Rad A5m Sepharose column, and the fraction containing larger cDNA molecules were collected and ligated to λgt10 or λgt11 arms. The DNAs were packaged into phage particles with a Gigapack plus kit and approximately 5 × 10<sup>5</sup> independent phase clones were obtained. The size of inserts in these clones was about 2 kb on average. This cDNA library was screened with *flt* genomic DNA probe using a method described by Benton & Davis (1977).

## DNA sequencing

Two overlapping cDNA clones, #3-7 and #3-5 were sequenced by the dideoxynucleotide method (Sanger *et al.*, 1977). To sequence both strands, these *flt* cDNAs were subcloned in pUC plasmid vectors in both orientations and

various deletions from the 5'- or 3'-end were introduced into the cDNA molecules by using a DNA deletion kit (Takara Shuzo, Kyoto). For DNA sequencing these deletion-containing plasmid DNAs were prepared and denatured to single-stranded form by an alkali method. The coding regions of the *flt* DNA were sequenced at least three times, including both directions.

## Northern blotting analysis

Total cellular RNA was prepared from various cell lines and tissues by a guanidine/cesium chloride centrifugation method (Chirgwin *et al.*, 1979). Poly (A)<sup>+</sup> RNA was obtained using oligo(dT) column chromatography and separated on formaldehyde-containing agarose gel (Lehrach *et al.*, 1977). RNA was transferred to a nitrocellulose filter, baked for fixation and hybridized with *flt* genomic or complementary DNA sequences. Hybridization condition was 3 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M Na citrate), 50% formamide at 37°C.

## Acknowledgements

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These sequence data will appear in the EMBL/GenBank/DBJ Nucleotide Sequence Databases under the accession numbers X51602.

COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

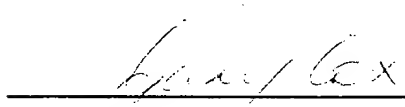
IN THE MATTER OF: Australian  
Patent Application 696764  
(73941/94). In the name of:  
Human Genome Sciences Inc.

- and -

IN THE MATTER OF: Opposition  
thereto by Ludwig Institute for Cancer  
Research, under Section 59 of the  
Patents Act.

Annexure GBC-9

This is **Annexure GBC-9** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
\_\_\_\_\_  
**Gary Baxter Cox**

WITNESS:

  
\_\_\_\_\_  
Patent Attorney

PAYTEE K. HOO

# Angiogenesis: Models and Modulators

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Angiogenesis *in vivo* is distinguished by four stages: subsequent to the transduction of signals to differentiate, stage 1 is defined as an altered proteolytic balance of the cell allowing it to digest through the surrounding matrix. These committed cells then proliferate (stage 2), and migrate (stage 3) to form aligned cords of cells. The final stage is the development of vessel patency (stage 4), generated by a coalescing of intracellular vacuoles. Subsequently, these structures anastomose and the initial flow of blood through the new vessel completes the process. We present and discuss how the available models most closely represent phases of *in vivo* angiogenesis. The enhancement of angiogenesis by hyaluronic acid fragments, transforming growth factor  $\beta$ , tumor necrosis factor  $\alpha$ , angiogenin, okadaic acid, fibroblast growth factor, interleukin 8, vascular endothelial growth factor, haptoglobin, and gangliosides, and the inhibition of the process by hyaluronic acid, estrogen metabolites, genestein, heparin, cyclosporin A, placental RNase inhibitor, steroids, collagen synthesis inhibitors, thrombospondin, fumagellin, and protamine are also discussed.

KEY WORDS: Angiogenesis, Cell proliferation, Cell migration, Proteolytic balance, Collagen synthesis inhibitors.

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## I. Introduction

Endothelial cells are derived from pluripotent mesodermal precursors during the process of vasculogenesis, which occurs in the extraembryonic

mesoderm of the yolk sac in both avian and mammalian embryos, and in selected organ systems (Risau and Lemmon, 1988; Pardanaud *et al.*, 1989). Angiogenesis is the development of the complex network of blood vessels that occurs following vasculogenesis, when endothelial cells proliferate and migrate throughout the embryo. The process of angiogenesis is important not only during embryological development, but during a variety of normal and pathological conditions in the adult, including ovulation, implantation, during mammary gland changes associated with lactation, bone formation, inflammation wound repair (Jakob *et al.*, 1977; Gospodarowicz and Thakral, 1978; Nomura *et al.*, 1989; Brannström *et al.*, 1988; Knighton *et al.*, 1990), and tumor growth (Folkman, 1985; Furcht, 1986).

Light and electron microscopy studies, combined with *in situ* hybridization, of both the normal genesis of vessels during embryological development and during tumor angiogenesis have demonstrated a number of discrete events that occur during angiogenesis (Schoeffl, 1963; Yamagami, 1970). Following a stimulus for neovascularization endothelial cells change their morphology and begin to degrade their surrounding basement membrane (Ausprunk and Folkman, 1977; Moscatelli *et al.*, 1980; Gross *et al.*, 1983). These "leading" cells must modulate the expression of their proteases to allow degradation of existing extracellular matrix (ECM) components, and the migrating cells following this front must be supported by the appropriate ECM to allow for their proliferation, migration, and differentiation into vascular tubes. This initial migration and proliferation is in a fibronectin-rich ECM, and during the later stages of angiogenesis, when cords of endothelial cells align, the cells express laminin, a matrix component associated with vascular maturation (Risau and Lemmon, 1988). Finally, the generation of vessel patency is achieved by the coalescing of intra- and intercellular vacuoles (Sabin, 1920; Lewis, 1925; Clark and Clark, 1937).

The dependence of tumor growth on angiogenesis is well documented (Folkman, 1990). This relationship has been demonstrated for many types of tumor, invasive breast cancer (Weidner *et al.*, 1991), non-small cell lung cancer (Macchiarini *et al.*, 1992), and prostate carcinoma (Weidner *et al.*, 1993). Studies using the pancreatic  $\beta$  cells of animals transgenic for a hybrid oncogene (RIP1-Tag2) (Brinster *et al.*, 1993) would indicate that angiogenesis is an important step in carcinogenesis in this system (Folkman *et al.*, 1989a).

To investigate factors that influence angiogenesis and to gain a more fundamental understanding of the cellular processes involved in the generation of capillaries, it has been necessary to develop a number of models of angiogenesis.

## II. Models of Angiogenesis

### A. Chicken Chorioallantoic Membrane Assay

The chicken chorioallantoic membrane assay is a technique traditionally used by embryologists that involves analysis of the developmental potential of grafts transplanted onto the chorioallantoic membrane (CAM). Because the early chicken embryo lacks a complete immune system xenografts from mammalian species become established and grow. Vascularization of these grafts is rapid.

Sorgente and colleagues (1975) first described the inhibitory effects of cartilage grafts on vascular development using this model. Subsequently, Folkman and co-workers (1979) used the model to study tumor angiogenesis directly. Fertile eggs were incubated for 72 hr and prepared for grafting by removal of enough albumin to facilitate the placement of a graft without causing subsequent cramping and sticking to the shell membrane. A rectangular window was cut in the shell to place and access the graft or test substance on the CAM. Angiogenesis was scored 3–4 days after grafting. Angiogenesis was considered to have been induced if a spoke-wheel arrangement of vessels was generated, directed toward the graft. Substances were lyophilized onto coverslips, then applied to the CAM to examine the effects on angiogenesis (Folkman *et al.*, 1979).

Quantitation of angiogenesis using the CAM assay was initially done on a graded score of 0–4, by observation. Computer analysis was subsequently applied to score the total number of vessels and obtain a directional vector value (Voss *et al.*, 1984; Jakob and Voss, 1984). The use of labeled sulfate to follow the angiogenic process has also made quantitation more accurate (Spisni *et al.*, 1992). Apart from problems associated with quantitation, the most common problem is the result of false positives due to wounding or irritants generated during the initial setting up of the assay. Because an angiogenic response may be consequent to wound healing or inflammation (Mahaderan *et al.*, 1989), this problem is not surprising. The CAM assay is sensitive to modification by many factors, including gas content and pH. The most pronounced variation observed is of keratinization, which in turn has significant effects on the CAM response to stimulation (Ausprunk *et al.*, 1991). This method has been applied to a wide range of both inhibitors and inducers of angiogenesis, as discussed in subsequent sections of this article (Folkman and Klagsbrun, 1987).

A further development of this model has been the *in vitro* method of maintaining the chick embryo in culture (Auerbach *et al.*, 1974). Although this is an *in vitro* assay, it is closest to a whole animal assay because the



entire embryo and its membranes remain intact. In this assay, the egg content is transferred to a petri dish, where development continues to take place. This model has the advantage that multiple grafts can be placed on one embryo, and the effects can be photographed over time. Quantitation is simplified by the fact that the *in vitro* CAM presents a two-dimensional monolayer, not subject to the distortion of the *in ovo* CAM assay. The advantage is that multiple grafts may then be placed on the one embryo, and they can more easily be photographed over time. A further modification of the *in vitro* CAM assay, in which the embryo is supported on Gladwrap stretched across the mouth of a beaker (Dunn *et al.*, 1981), has improved embryo survival. The advantages of increased viability are offset by the difficulty in photographing the results. This model is technically easier than the *in ovo* assay and is better suited to large-scale experiments. The addition of sterile silicon rings on the yolk sac membrane creates discrete observation windows and assists in quantitation (Takigawa *et al.*, 1990).

#### B. Corneal Neovascularization Model

As the cornea is normally avascular, induction of an angiogenic reaction is a true demonstration of neovascularization (Hendkin, 1978). The earliest studies of corneal neovascularization were in the rabbit (Gimbrone *et al.*, 1974), in which insertion of tumor cells or extracts placed within 2 mm of the cornea-scleral junction generated vascular sprouts within 36 hr. However, because of the absence of genetically similar strains, expense, and difficulty in handling, other species have been used for angiogenesis studies in the cornea, including guinea pigs, rats, and mice (Fournier *et al.*, 1981; Muthukkaruppan and Auerbach, 1979; Muthukkaruppan *et al.*, 1982). Although the use of mice overcame the strain variation problem their small size makes the introduction of slow-release polymer into the eye a procedure requiring microsurgical skill. Quantitation of corneal neovascularization is difficult owing to the variability arising from an inability to achieve uniform placement of the test substance. Consequently, reagents under test have been incorporated into ethylene-vinyl acetate pellets (Elvax) prior to implantation into the cornea (Gimbrone *et al.*, 1974; Risau, 1986). The implantation of tumor cells also requires the incorporation of those cells into an inert medium that allows for accurate placement (Ausprunk and Folkman, 1977). The expression of corneal-derived cytokines such as interleukin 8 (IL-8), which has been shown to be angiogenic, may also lead to some variability in assays of angiogenic factors (Strieter *et al.*, 1992).

Advances in image analysis (Proia *et al.*, 1988; Haynes *et al.*, 1989) have improved the capacity to quantitate using the corneal model. Often a computerized digitizer, for example the Optomax Image analysis system (Optomax, Hollis, NY) or similar, is used. This system consists of a high-sensitivity closed circuit television (CCTV) camera mounted on a Nikon Optiphot-2 microscope. The image is displayed on a color video monitor that is interfaced with a microprocessor. Histological slides stained with von Willebrand factor antibodies may be used to locate blood vessel formation. Sequential monitoring of neovascularization in individual animals makes it possible to evaluate progressive changes in the process (Folkval, 1991). Indeed, development of computer-assisted image analysis has made many models of angiogenesis more quantitative (Parke *et al.*, 1988).

### C. Pouch Assays

The hamster cheek pouch is considered to be an "immune privileged" site because allogeneic or xenogeneic grafts may grow without eliciting an immune response. The anterior eye chamber is another "immune privileged" site that has been used to study neovascularization of preneoplastic mammary tumor cells (Folkman *et al.*, 1989b). Quantitation of this model is by morphometric analysis of histologically prepared sections following angiogenesis. Tumor implants have also been used (Auerbach *et al.*, 1976) in this model, as have slow-release vectors to assess the effects of transforming growth factor (TGF- $\alpha$ ) (Schreiber *et al.*, 1986).

The dorsal air sac method was developed by Selye (1953), to monitor vascularization of tumor grafts. Dorsal air sacs are created by injecting 10–15 ml of air into the backs of rats, and the model modified by the insertion of a transparent window in the skin, through which the process may be monitored. Using this model, angiogenesis mediated by the injection of tumor cells (Sakamoto *et al.*, 1991) or endothelial cells (Schweigerer *et al.*, 1992) has been assayed in response to various reagents.

The method of subcutaneous implantation of polyvinyl acetate (PVA) sponge disks impregnated with angiogenic factors is in common use (Fajardo *et al.*, 1988). Flat sponges of PVA foam are cut into 11-mm disks and their flat sides are sealed with Millipore (Bedford, MA) filters. Prior to sealing, a core is cut where the test material is to be inserted. This core is sealed with a slow-release polymer, ethylene–vinyl acetate copolymer (Elvax) (Langer *et al.*, 1980), then reinserted into the sponge. The sponges are recovered 1–3 weeks after subcutaneous implantation. Xenon clear-

ance has been shown to be a useful means of quantifying new blood vessel formation (Andrade *et al.*, 1987).

Several *in vivo*, or *in ovo*, angiogenesis assays rely on being able to deliver a discrete amount of effector substance or cells to a precise location. Currently reagents are imbedded in Elvax, and the rate of release of components is dependent on the thickness of the coating of Elvax, making it difficult to reproduce these inserts. Alginate, a glycuron extracted from brown seaweed algae, gels in the presence of calcium ions or other multivalent counterions by anisocooperatively forming junctions between contiguous blocks of  $\alpha$ 1,4-L-glucuronan residues present in the polysaccharide. Growth of avian and mammalian chondrocytes in ionotrophically gelled alginate beads demonstrates the potential of using this model for an alternative delivery system in angiogenesis models (Guo *et al.*, 1989), or it may provide an alternative method for the slow release of effectors of angiogenesis (Downs *et al.*, 1982). Matrigel can also be injected subcutaneously in mice, and used as a vehicle to assess angiogenic activity of different compounds (Passanti *et al.*, 1992; Kibbey *et al.*, 1992). Although the subcutaneous injection of Matrigel alone is insufficient to induce focal angiogenesis when fibroblast growth factor (FGF)-heparin is mixed with the Matrigel, in-growth of vessels is observed within days. The Matrigel plug can be removed, and processed for vessel quantitation (Passanti *et al.*, 1992; Kibbey *et al.*, 1992).

#### D. Mesenteric Window Assay

The mesenteric window assay examines the effect of reagents on normally vascularized mammalian tissues. Angiogenesis in this model is mediated by autologous mast cells, and probably occurs frequently because mast cells are activated in tissue trauma, wound healing, inflammation, as well as in many clinical and experimental tumors (Enerback and Norrby, 1989). Although the mechanism of the mast cell-mediated angiogenic reaction is not completely understood it is known that preformed mast cell products such as heparin and histamine can be angiogenic (Norrby *et al.*, 1986, 1990; Garrison, 1990; Norrby and Sorbo, 1992; Sorbo and Norrby, 1992). Mast cell-mediated angiogenesis has also been reported using the CAM assay (Clinton *et al.*, 1988; Duncan *et al.*, 1992).

The mesenteric window assay is well suited to quantitative analysis. In addition to the number of vessels per unit length of tissue and the vascularized area, it permits quantitation of vascular density and total vascular quantity, as well as measurement of the branching pattern (Norrby *et al.*, 1990; Jakobsson and Norrby, 1991).

### E. Spontaneous Angiogenesis

When endothelial cells are maintained as a confluent monolayer for prolonged periods of time without replenishing the nutrients, capillary-like vessels spontaneously form. This "spontaneous" tube formation takes between 10 and 14 days after confluence (Folkman *et al.*, 1979). During spontaneous tube formation of human umbilical vein endothelial cells (HUVECs) the majority of the cells are involved in chaotic cell death, as nutrients become depleted. The capillary-like vessels generated from the small percentage of the population that differentiates are anchored loosely in the culture dish, with no formation of a monolayer (see Fig. 1). However, endothelial cells of bovine origin spontaneously form capillary-like tubes by a process of "sprouting" of a subpopulation of cells that form a reticular network of vessels sitting on top of a monolayer of cells (Maciag *et al.*, 1982). The reason for this difference is not understood but may relate to the fact that bovine endothelial cells have a lower growth factor requirement and may tolerate longer periods of time in growth factor-depleted culture conditions, suggesting that the cell death seen in the human cell cultures may be consequential and not important to angiogenesis in this model. An important feature in both cases is that only a small proportion of the initial cell population is involved in forming these tubelike structures. What distinguishes this small subpopulation, and at what point in the proceedings these cells commit to differentiation, remains unknown. In bovine aortic endothelial cell spontaneous tube formation, sprout formation precedes the generation of a capillary-like network on top of the monolayer, and has been shown to involve the synthesis of type I collagen (Cotta-Pereira *et al.*, 1980). The possibility of modulating this early event has come to light from studies in which the addition of 10–100  $\mu\text{g/ml}$  of type I collagen plus phorbol 12-myristate 13-acetate (PMA) to monolayers of human neonatal foreskin capillary endothelial cells rapidly (3 hr after addition) induced the initial sprouting patterns previously observed only in bovine spontaneous tube formation (Jackson and Jenkins, 1991). Further reports on this intriguing model have not been forthcoming.

We have observed that the rate of spontaneous tube formation is increased when confluent cultures of endothelial cells are stressed by altered pH or temperature (G. W. Cockerill, unpublished observation). This observation is consistent with the stress-related changes in heat shock protein 90 (hsp90), which have been shown to be mediated through alterations in extracellular matrix (Ketis *et al.*, 1993). Little is known about the effects of modulation of hsp90 on angiogenesis. However, because hsp90 has been shown to mediate the nuclear translocation of the estrogen receptor it is interesting to propose this as a productive avenue of research.



FIG. 1 Spontaneous angiogenesis of HUVECs. Endothelial cell monolayers were allowed to exhaust the culture medium over a period of 10–14 days. Although most of the cells died a small subpopulation differentiated to form tubelike structures. These structures have lumena, as determined by serial sectioning, and were attached to the edge of the culture dish, and also at a single point in the center of the dish (middle).

## F. Three-Dimensional Gel Assays

Several three-dimensional gel assays have been developed, including collagen type I (Montesano and Orci, 1985, 1987; Montesano *et al.*, 1986), fibrin (Montesano *et al.*, 1987), fibronectin (Ingber and Folkman, 1989a), and Matrigel (Kubota *et al.*, 1988). Here we discuss the collagen and Matrigel model.

### 1. Type I Collagen Gels

The addition of PMA to endothelial cells seeded onto type I collagen induces invasive, capillary-like tubes (Montesano and Orci, 1985; Montesano *et al.*, 1987), as illustrated in Fig. 2, suggesting involvement of protein kinase C (PKC) in this process. Invasion is mediated by inducing expression and synthesis of type I collagenase, plasminogen activator (PA) activity, and stromelysin in endothelial cells in culture (Gross *et al.*, 1982; Moscatelli and Rifkin, 1988; Herron *et al.*, 1986). Kalebić and

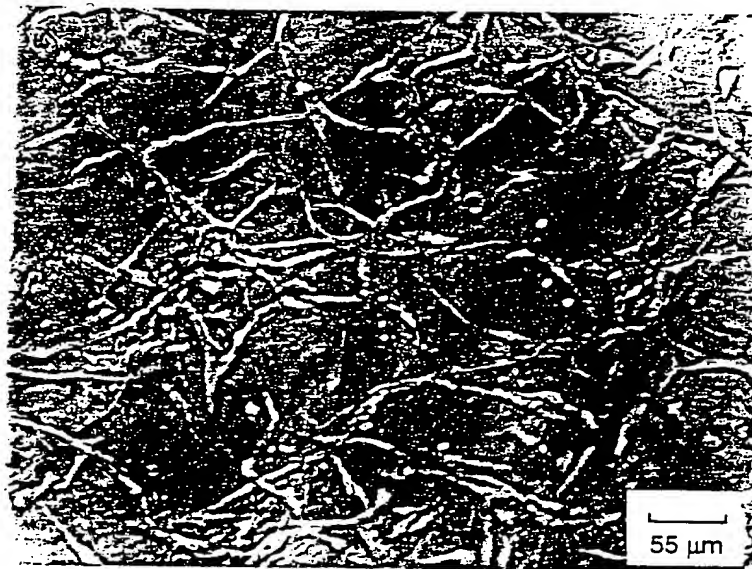


FIG. 2 Collagen gel model of angiogenesis. Capillary-like vessels were generated within 24 hr of seeding human umbilical vein endothelial cells onto thick type I collagen gels in the presence of PMA.

colleagues (1983) have also shown type IV collagenase is present in endothelial cells. Although these metalloproteinases are likely to be produced in their latent/inactive forms, it is possible that they are activated locally by plasmin produced by the action of coordinately expressed urokinase-type plasminogen activator (uPA).

Reduction of proteolytic activity may be achieved by several protease inhibitors. Plasminogen activator inhibitor 1 (PAI-1) is the major secreted inhibitor of bovine aortic endothelial cells (van Mourik *et al.*, 1984), and has been shown to be expressed in virtually all endothelial cell types (Moscatelli and Rifkin, 1988). Tissue inhibitor of metalloproteinases (TIMP) has been shown to be synthesized by rabbit brain capillary endothelial cells, and is able to inhibit collagenase, stromelysin, and other proteases (Herron *et al.*, 1986). Another level of control of angiogenesis may be afforded by the differential localization of expression of proteases and inhibitors, whereas the proteases are often cell surface associated, the PAI-1 is ECM associated, and TIMP may be secreted into the interstitial fluids.

Comparison between endothelial cell invasion and tumor cell invasion suggests that the proteolytic activity of endothelial cells may be cell associated (Moscatelli and Rifkin, 1988). This hypothesis is supported by the fact that uPA (Moscatelli, 1986), plasminogen, and plasmin bind to cultured endothelial cells (Bauer *et al.*, 1992). Other components of the proteolytic cascade have also been localized to endothelial cell ECM. For example, PAI-1 is associated with the substratum and stabilized in its active form such that it can complex with tissue-type plasminogen activator (tPA) and inhibit its activity (Levin and Santell, 1987). Plasminogen activator inhibitor 1 deposited near endothelial cells may protect the capillary basement membrane and other matrix proteins from proteolysis by plasmin-generating enzymes. In addition, enzyme activation may be favored by the localized deposition of proenzymes and activators, which could result in an enhanced rate of enzyme activity and invasion. Protection from inactivation from secreted or ECM-associated inhibitors, and localization of enzyme activity to discrete regions allow proteolysis and thus angiogenesis to proceed in a specific direction (Moscatelli and Rifkin, 1988).

Tube formation in this model occurs within 24 hr and requires both transcription and translation (Montesano and Orci, 1985). Cells are seeded onto the top of a thick gel of collagen in the presence of PMA. The subsequent cell invasion of the matrix mimics the early events seen during angiogenesis *in vivo*. Capillary-like structures form throughout the gel, making quantitation difficult. Not all cells are stimulated to differentiate, and some remain as a monolayer on the surface of the gel.

## 2. Matrigel

The basement membrane is an important biological mediator of angiogenesis, and has been exploited in both *in vivo* and *in vitro* assays to assess the angiogenic activity of various factors (Madri *et al.*, 1983; Ingber and Folkman, 1989a,b; Form *et al.*, 1986). Matrigel is made by extracting the basement membrane matrix of Englebreth-Holm-Swarm (EHS) tumors taken from lathrytic mice (Kleinman *et al.*, 1982). At 4°C the extract is a viscous liquid that gels on warming to 37°C. The major components of this material are laminin, collagen IV, entactin/nidogen, heparan sulfate proteoglycan (Kleinman *et al.*, 1982), and growth factors (Taub *et al.*, 1990). The direct application of the material to angiogenesis was by Kubota and colleagues (1988). Figure 3 shows a typical response of human umbilical endothelial cells to this matrix. Within 1 hr the cells have rapidly migrated into a reticular network of aligned cells (Fig. 3a), after 2 hr the cells have started to flatten (Fig. 3b), and by 12–18 hr they have formed a network of capillary-like structures on the surface of the gel (Fig. 3c). These structures have a well-defined lumen that can be visualized by serial cross-section at the electron microscope level. Tube formation on Matrigel is a density-dependent phenomenon (Fig. 4). At too high a cell density a monolayer is formed, and at too low a cell density the cells do not contact each other, and in both instances tube formation is inhibited (Cockerill *et al.*, manuscript in preparation).

Alignment of the cells appears to be necessary for tube formation on Matrigel. However, many cell types are able to transiently form an aligned network on top of the Matrigel gels (Emonard *et al.*, 1987), but do not form structures with a lumen, indicating that alignment is necessary but not sufficient for tube formation. Figure 5 shows the time course of tube formation for HUVECs (Fig. 5, left) and a stromal fibroblast cell line (Fig. 5, right). Although the cells appear to align (Fig. 5A and B), only the HUVECs remain in the reticular pattern 2 hr after seeding (Fig. 5C), whereas the stromal cells are clumping together in nodules (Fig. 5D). Whereas the HUVECs still display a network of capillary-like vessels after 24 hr (Fig. 5E), the stromal cells are in tight nodules (Fig. 5F). The inset shows the stromal cells 3 days after seeding, at which time the cells begin to migrate out of the nodules as solid cords of cells (Cockerill *et al.*, manuscript in preparation). Matrigel seems to support differentiation of many cell types. Mammary epithelial cells form nodes that produce casein (Seeley and Aggeler, 1991), and baby mouse kidney cells form nodes that eventually (after 6 days) form structures with lumina (Klein *et al.*, 1988). Sertoli cells form short, cordlike structures (Hadley *et al.*, 1990).

Alignment of endothelial cells on Matrigel does not require protein synthesis or gene expression (Bauer *et al.*, 1992). However, tube formation



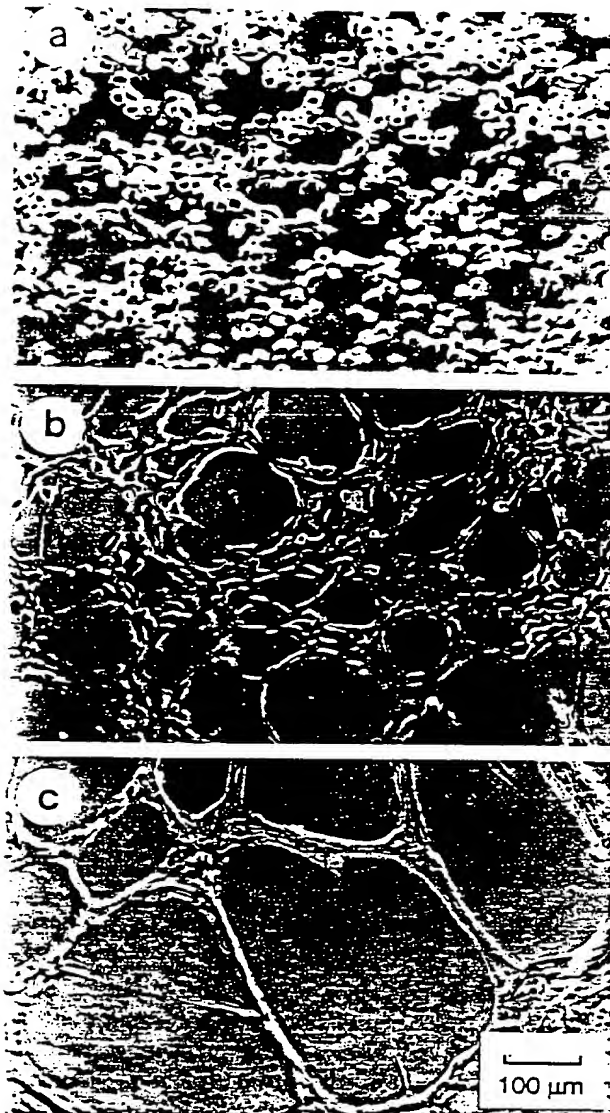


FIG. 3 Matrigel model of angiogenesis. HUVECs align within 60 min of being seeded onto thick Matrigel gels (a), and by 2–3 hr have flattened into a reticular network on the surface of the gel (b), and within 12–20 hr the cells have formed a mesh of capillary-like vessels (c).

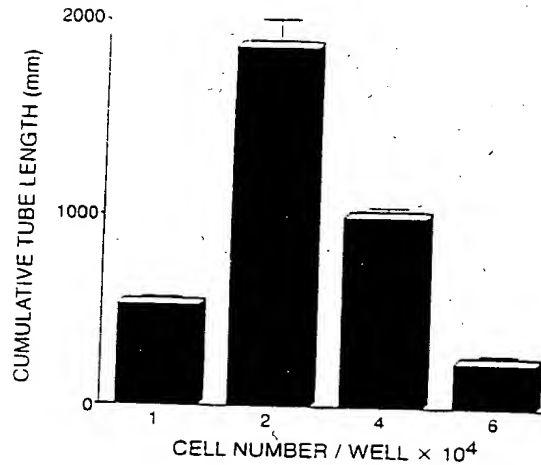


FIG. 4 Angiogenesis on Matrigel is density dependent. The extent of tube formation in HUVECs seeded onto Matrigel gels over a range of cell densities, was measured. The optimal cell density, using gels in microtiter trays, was  $2 \times 10^4$  cells/well. At the highest density examined ( $6 \times 10^4$  cells/well) we observed an approximately 6-fold reduction in angiogenesis. At the lowest cell density examined, the number of capillary-like vessels was reduced approximately 3.5-fold.

does require gene expression during the period of cell alignment, as the addition of transcriptional inhibitors during alignment abolishes tube formation whereas the addition of inhibitors after this event does not affect tube formation (Fig. 6). Gene induction by Matrigel has also been demonstrated in HUVECs (Sarma *et al.*, 1992), where contact with the matrix induces a primary response gene.

As in the type I collagen assay PKC mobilization is clearly required in the Matrigel model of angiogenesis (Bauer *et al.*, 1992; Kinsella *et al.*, 1992). Unlike the collagen gel model, the activation of PKC observed on Matrigel does not lead to invasion of the cells into the gel, suggesting that the balance of proteolytic enzymes is significantly different between the two models. The Matrigel model of angiogenesis more accurately represents conditions that are likely to occur as a late event in angiogenesis *in vivo*. Differentiation of endothelial cells on Matrigel may be blocked by pertussis toxins, indicating that the process requires G proteins (Bauer *et al.*, 1992). These cAMP-dependent G proteins have been shown in other systems to be important in cell-cell interactions and development (Devrotes, 1989). It remains to be seen if the G protein-coupled receptor cloned by differential screening of endothelial cell libraries with and without PMA activation (Hla and Maciag, 1990) has a role in angiogenesis.

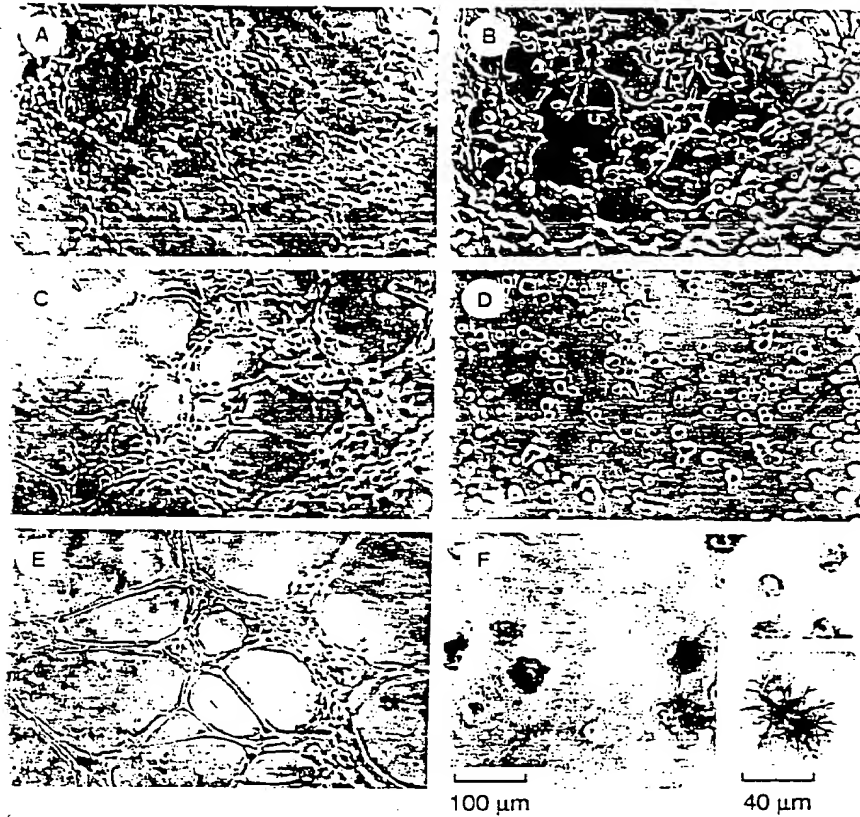


FIG. 5 Tube formation on Matrigel is cell specific. *Left:* The time course of angiogenesis seen in normal HUVECs. *Right:* The time course of events when stromal fibroblasts are seeded onto Matrigel under the same conditions. After 1 hr both cell types appear to have aligned (A and B). At 2 hr the HUVECs are flattening (C), whereas the stromal fibroblasts are clumping together (D). At 20 hr postseeding, the HUVECs have formed a reticulum of vessel-like structures (E) whereas the fibroblasts have aggregated into dense balls (F). Interestingly, at 3 days the stromal fibroblasts were beginning to migrate out of the cell clusters (inset). Serial sectioning of these structures showed only cords of cells, with no apparent lumen.

Similar to other models of angiogenesis, the Matrigel-induced angiogenesis may be inhibited using analogs of proline to inhibit collagen synthesis (Grant *et al.*, 1991).

Unlike the collagen gel model of angiogenesis the majority of cells seeded onto Matrigel gels will differentiate and enter into angiogenesis. The basement membrane extract of the EHS sarcoma was the starting

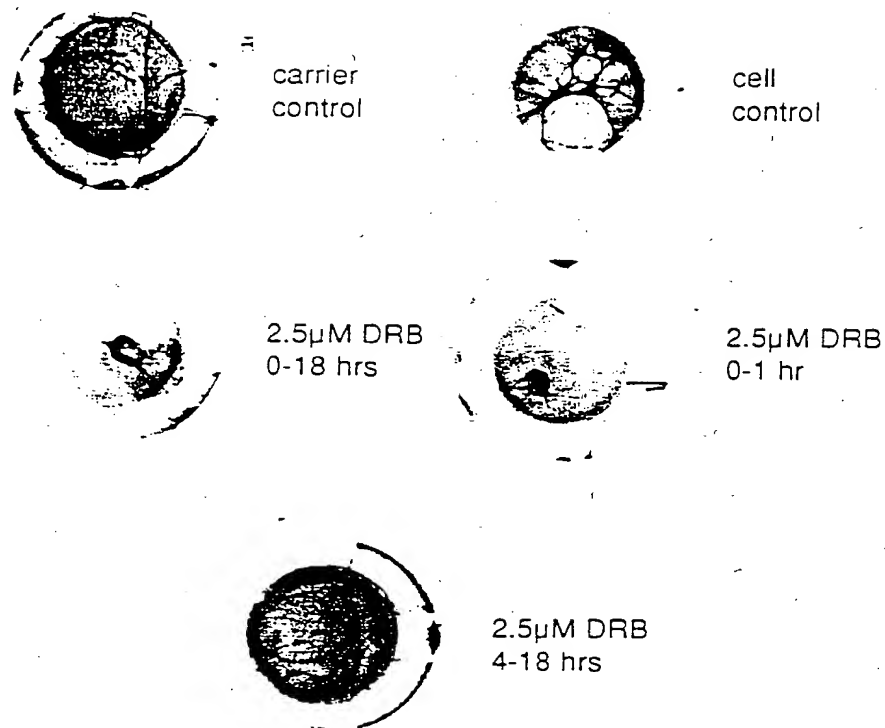


FIG. 6 Angiogenesis on Matrigel requires gene expression. HUVECs were plated onto Matrigel and allowed to form capillary networks. By using the reversible transcriptional inhibitor (DRB) we were able to show that inhibition of transcription during the first hour, although not affecting alignment of the cells, completely abolished the development of capillary-like structures. The addition of the inhibitor after alignment and flattening had no effect on the ability of the cells to form capillary-like structures.

material for the purification of the calcium-binding basement membrane protein 40 (BM-40)/SPARC (Dziadek *et al.*, 1986). Although the steady state levels of SPARC mRNA have been shown to increase during spontaneous tube formation, levels do not change during angiogenesis on Matrigel. Because SPARC has been shown to be able to arrest cells in cycle (Funk and Sage, 1991) it is interesting to suggest that this may be a mechanism for establishing synchrony in this model, explaining why almost all the cells on Matrigel differentiate.

Matrigel is subject to batch-to-batch variation. It is likely that small variations in components greatly affect cell adhesion, motility, and proliferation, and all contrive to alter the differentiation of endothelial cells on Matrigel. We have observed differences in adhesion and also in levels and types of proteoglycan between batches. An altered combination of matrix components may lead to differences in the malleability of the final gel. Studies indicate that this is a factor that could potentially alter cell response (Vernon *et al.*, 1992). One of the advantages of its use is that the cell response is more homogeneous and, because the capillary-like structures generated form on the surface of the gel, the model is relatively easy to quantitate (Grant *et al.*, 1989).

To date, the factors tested in *in vitro* and *in vivo* Matrigel assays show activities similar to those observed in the CAM model (Grant *et al.*, 1989; Sakamoto *et al.*, 1991; Passanti *et al.*, 1992; Kibbey *et al.*, 1992).

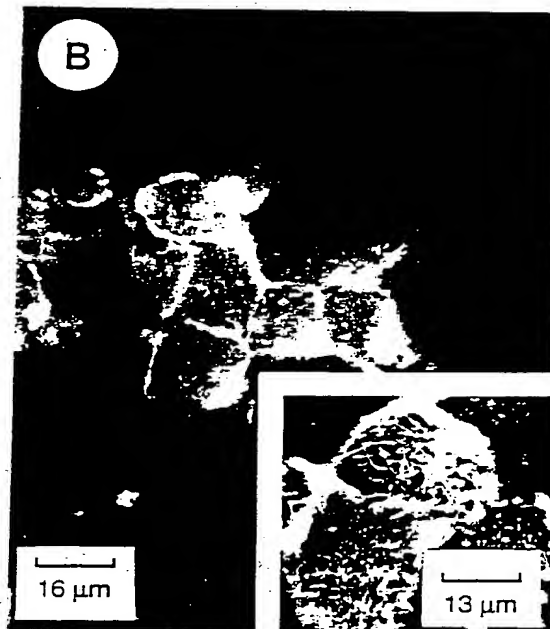
### 3. Future Directions

Laminin antibodies that block binding of endothelial cells to laminin or Matrigel demonstrated a requirement for cell adhesion prior to growth and differentiation (Grant *et al.*, 1989). From these studies it was also suggested that the rapidity of angiogenesis on Matrigel versus collagen gels was a result of the need to synthesize a basement membrane on collagen gel that was already present on Matrigel (Grant *et al.*, 1989). Application of YIGSR-NH<sub>2</sub> laminin peptide to a monolayer of endothelial cells resulted in 30% of the population developing a ringlike structure, suggested by the authors as paralleling lumen formation (Grant *et al.*, 1989). Could this be a significant way of distinguishing a tube-competent endothelial subpopulation? If this suggestion is supported then this phenomenon would be appropriate to apply to subtractive hybridization, or the more recently developed differential display technology to clone those genes that mediate these events.

A number of workers have previously suggested a role for integrins in the *in vitro* angiogenic behavior of endothelial cells on extracellular matrix (Grant *et al.*, 1989; Basson *et al.*, 1990). The addition of monoclonal antibodies against  $\alpha 6$  and  $\beta 1$  to endothelial cells seeded onto Matrigel completely blocked angiogenesis on this matrix (Bauer *et al.*, 1992). Because laminin is the major component of this gel and the  $\alpha 6 \beta 1$  integrin is shown to be the major receptor for laminin (Sonnenberg, 1988), it is likely that antibodies prohibit cell attachment to such a degree as to prevent the formation of capillary-like structures. We have shown that this model of angiogenesis is density dependent (Fig. 3), therefore it is not clear if inhibition of cell adhesion to Matrigel per se blocks angiogenesis, or whether by reducing the cell number finally attached to the gel (Bauer *et*

*et al.*, 1992), they have inhibited angiogenesis by reducing the cell density. In similar studies, polyclonal antibodies to the entire vitronectin ( $\alpha v\beta 3$ ) and fibronectin ( $\alpha 5\beta 1$ ) receptors totally inhibited cell adhesion to Matrigel and hence capillary formation in Matrigel (Davis *et al.*, 1993). The use of  $\alpha 5$ -integrin monoclonal antibodies in the same studies either had no effect or enhanced tube formation (Bauer *et al.*, 1992; Davis *et al.*, 1993). A clue to the mechanism of this effect comes from a study by Gamble *et al.* (1993), in which selectively restricting the adhesive repertoire of endothelial cells for the specific matrix to which they are exposed, the authors were able to show an enhancement of angiogenesis. On collagen gels in the presence of PMA,  $\alpha 2\beta 1$  antibodies that block the collagen receptor enhanced tube formation, whereas no effect was seen on fibrin gels (Gamble *et al.*, 1993). Conversely, antibodies directed against the major fibrin receptor  $\alpha v\beta 3$  enhanced tube formation on fibrin gels, but had no effect on collagen. Thus, restricting the usage of fibronectin receptors ( $\alpha 5\beta 1$ ) on Matrigel by the use of anti- $\alpha 5$  antibodies may explain the enhancement observed in the Bauer *et al.* studies (1992). The potential use of anti-integrin antibodies as therapeutic agents has recently been demonstrated in the CAM assay using anti- $\alpha v\beta 3$  antibodies (Brooks *et al.*, 1994).

Matrigel induces a motile phenotype in endothelial cells seeded onto the gel. Within 5 min of contacting the gel the endothelial cell is covered with microspikes that may be visualized by fluorescently labeled phalloidin stains (Fig. 7). Whereas on plastic the endothelial cytoskeleton rapidly polymerizes and forms a complex network of filaments observable after 20–30 min, after several hours on Matrigel the only cytoskeletal architecture one can detect with phalloidin is at cell junctions and in the microspikes on the cell surface. Not until late in tube formation on Matrigel can actin filaments, running parallel to the tube, be observed (Fig. 8). We postulate that the establishment of a motile phenotype is likely to be a prerequisite of endothelial cell differentiation. This proposal is supported by studies that show that the angiogenin-binding protein is a 42-kDa cell surface actin-related molecule (Hu *et al.*, 1991; Moroianu *et al.*, 1993). In addition, several other more potent angiogenic factors have actin-binding capacity and the angiogenin has been shown to be able to induce actin polymerization at suboptimal concentrations for spontaneous polymerization (Hu *et al.*, 1993). The fact that angiogenic factors such as basic FGF (bFGF) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) can stimulate angiogenesis by receptor-mediated mechanisms, but can also bind actin, suggests that actin binding may provide a more general mechanism for mediating angiogenesis. Alternatively, it may provide a mechanism for mediating an early event in the process, subsequent to which receptor-mediated mechanisms may play the major role. These observations may distinguish an early event in angiogenesis, which demands further research.



### G. Coculture Models of Angiogenesis

Angiogenesis *in vivo*, rarely occurs within an environment free of other cell types. The influence of other cell microenvironments on microvessel formation and the expression of tissue-specific endothelial properties are being increasingly realized (Auerbach *et al.*, 1987; Butcher *et al.*, 1980). In a more recent model, angiogenesis is induced by culturing brain capillary endothelial cells on collagen gels in a chamber above confluent tumor cells, such that both cell types are bathed in the same medium (Okamura *et al.*, 1992; Abe *et al.*, 1993). Other groups have been successful in the use of a variety of cell types such as esophageal cancer cells (Okamura *et al.*, 1992) and keratinocytes (Ono *et al.*, 1992) to induce angiogenesis.

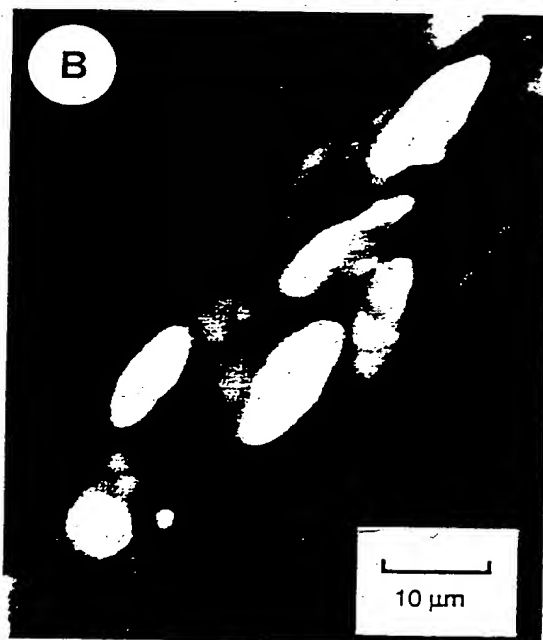
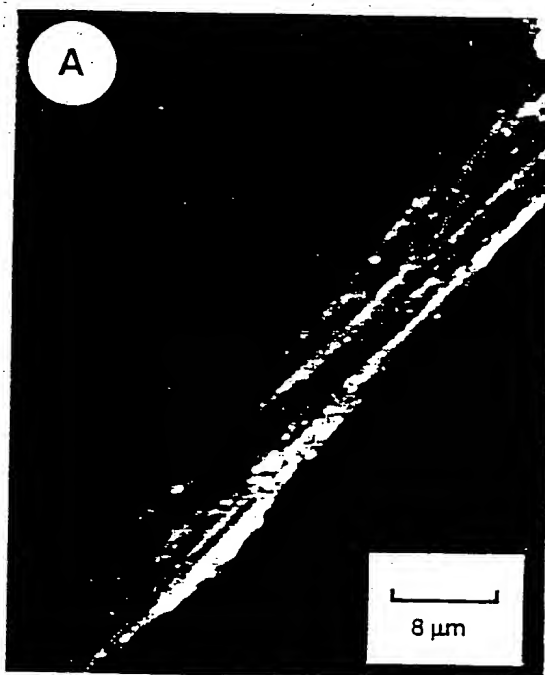
Demonstration of the abilities of astroglial cells to induce angiogenesis in brain capillary endothelial cells (Lattera *et al.*, 1990) has provided a model for the study of neural microvessel development, and blood-brain barrier formation. In this model, 24 hr after the seeding of C6 astroglial cells in a culture, endothelial cells are seeded at twice the density. Angiogenesis in this model requires both gene expression and protein synthesis (Lattera and Goldstein, 1991), and was induced within 24 hr of coculture. Furthermore, the induction of angiogenesis required direct cell-cell contact, as no enhancement was observed when the two cultures were bathed in the same growth medium in a Boyden chamber, where they were not in direct contact. Quantitation of this model is facilitated by being able to differentially stain the cell types involved in the culture model. In addition, computer-assisted analysis of fluorescently stained photographs enables the assay to be relatively accurate.

### H. Aortic Ring Model

The radial growth of microvessels is easily monitored in rings of aortas imbedded in three-dimensional (thick) gels, using standard phase microscopy. The end point can be histologically processed and sections cut for morphometric analysis (Nicosia and Ottinetti, 1990). Aortic rings of rat aorta embedded into collagen or fibrin gels in the absence of exogenous

FIG. 7 Actin staining of early events in angiogenesis on Matrigel. At optimal cell density on Matrigel, HUVECs aligned within 1 hr (see Fig. 3a). The cells were polarized end-to-end. Actin staining with Bodipy-phalloidin showed polymerized actin cytoskeleton at cell junctions and in microspikes (A). At high cell density, the cells attached to the matrigel as a monolayer and did not form extensive capillary-like structures. Actin staining of densely seeded cells after 1 hr (B) showed no end-to-end polarity and polymerized actin cytoskeleton surrounded most of the cells. The inset in (B) shows the staining of microspikes on the cells, characteristic of the motile phenotype.





growth factors can also generate a complex array of microvessels. This demonstrates the potential usefulness of this more defined model of angiogenesis (Nicosia and Ottinetti, 1990).

#### I. Human Amnion

The basement membrane of the human amnion may be used as a growth substrate for microvascular endothelium (Madri *et al.*, 1983; Furie *et al.*, 1984). Endothelial cells differentiate on this substrate but do not invade (Madri *et al.*, 1983; Furie *et al.*, 1984), as observed in the Matrigel model.

#### J. Summary

Whereas the collagen gel assay and the aortic ring model are best suited to investigating the effects of reagents on invasion of interstitial collagens, the human amnion and Matrigel model are best suited to examining the effects of mediators on later events in angiogenesis. All of these models address subtly different aspects of angiogenesis. To examine thoroughly the likely effects of a reagent, multiple models should be used. The *in vivo* model most appropriate for a particular reagent needs to be employed.

### III. Enhancers and Inhibitors

#### A. Enhancers and Angiogenesis

Classically, angiogenic factors have been defined as those that act directly on the endothelial cells to stimulate motility and mitosis, and as those that act indirectly to induce host cells to release growth factors that then target endothelial cells (Folkman and Klagsbrun, 1987). Development of reagents that enhance angiogenesis would have direct relevance in the management of severe wounds, and would facilitate many situations. Although some factors have been purified from highly vascular tumors, more

FIG. 8 Fluorescent staining of capillary-like structures on Matrigel. The parallel arrays of actin filaments can be observed in the capillary-like structures formed by HUVECs on Matrigel. (a) A capillary stained with Bodipy-phalloidin. (b) The nuclei of a tube stained with propidium iodide, demonstrating the multicellular nature of these structures.

recently a factor has been isolated from the blood of patients with systemic vasculitis (Cid *et al.*, 1993).

### 1. Transforming Growth Factor $\beta$

Transforming growth factor  $\beta$  is angiogenic *in vivo* (Roberts *et al.*, 1986). However, whereas TGF- $\beta$  induced tube formation when microvascular endothelial cells were placed in thick collagen gels (Madri *et al.*, 1988), it inhibited proliferation (Frater-Schroder *et al.*, 1986) and migration (Heinmark *et al.*, 1986) in other cell culture systems. Furthermore, in cultured bovine capillary endothelial cells TGF- $\beta$  decreased the amount of cell-associated and secreted PAI-1, decreasing cell invasion into collagen matrices and through amniotic membranes (Muller *et al.*, 1987; Mignatti *et al.*, 1989). Because TGF- $\beta$  is a potent chemoattractant for macrophages (Wahl *et al.*, 1987), it is possible that the TGF- $\beta$ -induced neovascularization is a consequence of angiogenic components produced from attracted macrophages.

### 2. Tumor Necrosis Factor $\alpha$

Antibodies to TNF- $\alpha$  have been shown to neutralize the angiogenic activity of thioglycolate-treated macrophages in the chick chorioallantoic membrane assay, and also in the type I collagen gel assay (Liebovich *et al.*, 1987), and in the rabbit corneal model (Frater-Schroder *et al.*, 1987). With both TNF- $\alpha$  and TGF- $\beta$  angiogenesis is associated with an inflammatory process, unlike bFGF-induced angiogenesis (Esch *et al.*, 1985).

### 3. Angiogenin

Angiogenin is a 14-kDa protein initially identified in HT 29 adenocarcinoma cells (Fett *et al.*, 1985). It was later found to be in adult liver, and at lower concentrations in many normal tissues as well as in serum (Weiner *et al.*, 1987; Shapiro *et al.*, 1987). Angiogenin shows 35% homology with pancreatic RNase (Shapiro *et al.*, 1987). However, its RNase activity is limited compared to pancreatic RNase, as it can break down tRNA, but only partially cleaves 18S and 28S ribosomal RNAs. Site-directed mutagenesis to determine the significant residues for RNase activity shows an increase in RNase activity with a concomitant loss in angiogenic activity (Shapiro *et al.*, 1989; Harper *et al.*, 1989). Further studies suggest that the RNase activity site in angiogenin is essential, but not sufficient, for its angiogenic activity, and that a second site on the molecule is also required (Hallahan *et al.*, 1991). Most recently, it was shown that the specific endocytosis of angiogenin is followed by nuclear translocation

(Moroianu and Riordan, 1994). Nuclear translocation has also been demonstrated to occur with the FGFs and endothelial cell growth factor (EGF) (Bouche *et al.*, 1987; Baldine *et al.*, 1990; Sano *et al.*, 1990), suggesting that this may be a common pathway in the mechanism of angiogenesis. Angiogenin can bind specifically to the endothelial cell and this binding is specifically inhibited by RNase inhibitor (Badet *et al.*, 1989). In addition, placental RNasin binds to angiogenin hundreds of times more efficiently than other RNases (Fox and Riordan, 1990). An angiogenin-binding protein with properties consistent with being an angiogenin receptor component has been identified in a transformed endothelial cell line, GM7373 (Hu *et al.*, 1993). It is a 42-kDa cell surface protein that is released by exposure of cells to heparin, heparin sulfate, or angiogenin. This protein has been shown to be a cell surface actin (Hu *et al.*, 1993). Angiogenin was able to induce the polymerization of actin at suboptimal concentrations required for spontaneous polymerization. This ability may be central to its mechanism of action, because such events could result in shape change and detachment, and precipitate subsequent events such as migration and proliferation, which lead to capillary formation. In support of this idea, reorganization of extracellular actin has been observed during the growth and formation of the corneal endothelium (Klagsbrun and D'Amore, 1991).

#### 4. Fibroblast Growth Factor

The heparin-binding fibroblast growth factor (FGF) family, acidic FGF (aFGF/FGF-1) and basic FGF (bFGF/FGF2), are among the growth factors that act directly on vascular cells to induce endothelial cell growth and angiogenesis (Burgess and Maciag, 1989; Ausprunk and Folkman, 1977; Abraham *et al.*, 1986; Gospodarowicz *et al.*, 1984; Thomas *et al.*, 1985; Yanagisawa-Miwa *et al.*, 1992). Whereas aFGF is found primarily in normal tissues (Risau *et al.*, 1988) and in vascular SMC (Winkles *et al.*, 1988), bFGF has a wide distribution (Lobb *et al.*, 1986; Schweigerer *et al.*, 1987). Because FGFs lack a signal sequence for secretion, their normal mode of release is not fully understood (Burgess and Maciag, 1989). Basic FGF is, however, associated with the ECM components, and its most important stored form is thought to be complexed to heparin sulfate proteoglycan (Folkman *et al.*, 1988). It has been hypothesized that poor perfusion in tumors may result in ischemia, acidosis, and tissue damage, which in turn may release FGF from the cells and ECM stores, and subsequently stimulate angiogenesis (D'Amore and Thompson, 1987).

Fibroblast growth factor may be detected after endothelial injury (Gadjusek and Carbon, 1989; McNeil *et al.*, 1989), and are present in the subendothelial matrix (Vlodavsky *et al.*, 1987). However, several other genes are observed to induce intimal hyperplasia, such as platelet-derived growth

factor (PDGF) or TGF- $\beta$  (Nabel *et al.*, 1993b). Studies using direct gene transfer of a secreted form of aFGF (Nabel *et al.*, 1993a) showed the induction of intimal thickenings 21 days after gene transfer. Neovascularization of this intimal thickening was observed, suggesting that the FGFs could potentially cause neovascularization in similar preatherosclerotic lesions. Acidic FGF stimulates hyperplasia and neovascularization of the hyperplastic intima, suggesting that smooth muscle hyperplasia alone is insufficient for the formation of new capillaries.

The locomotion of cells of endothelial origin is suppressed by TGF- $\beta$  but is enhanced by bFGF (Sato and Rifkin, 1988; Madri *et al.*, 1988). The locomotion of many normal and transformed cells of epithelial and mesenchymal origin can also be induced by members of the FGF family, insulin-like growth factor (IGF), PDGF, TGF- $\alpha$ , TNF- $\alpha$ , colony-stimulating factors (CSFs), interleukin 8 (IL-8), and interferons, in addition to complement and some matrix proteins (reviewed in Stoker and Gherardi, 1991).

Mignatti and colleagues (1989) have shown that bovine capillary endothelial cell migration through human amnion basement membrane was inhibited with antibodies to bFGF. This FGF-induced migration could also be inhibited by inhibitors of both plasmin and metalloproteinases as well as antibodies to tPA and type I and IV collagenase, demonstrating that both tPA, plasmin, and specific metalloproteinases are involved in the bFGF-induced invasion associated with angiogenesis.

### 5. Vascular Endothelial Growth Factor/Vascular Permeability Factor

The vascular endothelial growth factor (VEGF) family of proteins, also referred to as vascular permeability factor (VPF), exists as dimeric glycoproteins of  $M_r$  34K–46K that affect capillary permeability, and stimulate endothelial cell growth *in vitro*, and angiogenesis *in vivo* (Keck *et al.*, 1989; Connolly *et al.*, 1989; Ferrara *et al.*, 1992). Vascular endothelial growth factor has been characterized in several tumors of different species (Ferrara *et al.*, 1992), and is structurally related to PDGF with 18% identity between VEGF and the PDGF B chain. Vascular endothelial growth factor, like PDGF, can bind heparin and can be eluted off at low salt (Ferrara *et al.*, 1992; Senger *et al.*, 1990). Monoclonal antibodies to VEGF inhibited the growth of tumors in nude mice (Kim *et al.*, 1993). Four splice variants of VEGF may exist in four different homodimeric molecular species (Leung *et al.*, 1989; Houck *et al.*, 1991; Tisher *et al.*, 1991). A variety of transformed cell lines express the VEGF mRNA and secrete VEGF (Senger *et al.*, 1986; Rosenthal *et al.*, 1990). *In situ* hybridization studies demonstrate high levels of VEGF mRNA in highly vascularized glioblastoma multiforme and capillary hemangioblastoma (Berse *et al.*, 1992;

Shweiki *et al.*, 1992; Plate *et al.*, 1992; Berkman *et al.*, 1993). Monoclonal antibodies capable of blocking VEGF-induced angiogenesis *in vivo* and *in vitro* were used to assess the effect of VEGF on tumor growth (Kim *et al.*, 1992).

## 6. Interleukin 8

Interleukin 8 is a cytokine involved in leukocyte-vascular endothelial cell interactions such as the invasion of neutrophils through a vessel wall model via  $\beta 2$ -integrin attachment (Huber *et al.*, 1991), which more recently has been shown to have angiogenic properties (Koch *et al.*, 1992). It has also been implicated in angiogenic disease states such as psoriasis and rheumatoid arthritis (Brennan *et al.*, 1990; Seitz *et al.*, 1991; DeMarco *et al.*, 1991; and Schroeder and Christophers, 1989). Indeed, the rheumatoid synovium is a major source of IL-8. Similar to other angiogenic factors, IL-8 was shown to bind heparin and to have potent angiogenic activity when implanted into the rat cornea (Koch *et al.*, 1992). It also induced proliferation and chemotaxis of human endothelial cells (Koch *et al.*, 1992). However, whereas 2–40 ng of IL-8 induced corneal vascularization, 400 ng did not induce significant angiogenesis in this model, suggesting that in areas where high concentrations of IL-8 are produced (in areas of acute severe inflammation) neovascularization may not occur (Robbins *et al.*, 1984). Differing dose-dependent actions of IL-8 have also been demonstrated for neutrophil chemotaxis. Hence, high levels of IL-8 induce neutrophil chemotaxis (Yoshimura *et al.*, 1987; Larsen *et al.*, 1989) but low levels result in selective lymphocyte chemotaxis (Yoshimura *et al.*, 1987). In support of the idea that IL-8 may have a direct role as an inducer of neovascularization, studies have shown that recombinant IL-8 (rIL-8) can induce endothelial chemotaxis and proliferation (Koch *et al.*, 1992). These findings raise the possibility that TNF- $\alpha$ - or IL-1 $\beta$ -induced angiogenesis in the cornea may be mediated by induction of endogenous IL-8. Also, other factors may be produced in the cornea that mediate angiogenesis. For example, bFGF may be released from corneal extracellular matrix by the action of heparitinase (Folkman *et al.*, 1988), which may be important in mediating corneal repair.

## 7. Phosphatase Inhibitors

Vanadate potentiates the effect of growth factor-induced angiogenesis (Spisni *et al.*, 1992). Okadaic acid also induces angiogenesis in the chick chorioallantoic membrane model, with a minimum effective dose of 5 fmol/egg, and half-maximal dose being 90 fmol/egg (Oikawa *et al.*, 1992). Okadaic acid exerts an angiogenic activity an order of magnitude stronger than PMA but the time course of induction is slower than for PMA.

suggesting a differing mechanism of action (Suganumu *et al.*, 1988). Okadaic acid inhibits type 1 and 2A protein phosphatases, resulting in an increase in phosphoproteins within the cell (Haystead *et al.*, 1989; Sassa *et al.*, 1989; Yatsunami *et al.*, 1991). Unlike PMA, okadaic acid stimulates the production of prostaglandin  $E_2$  in rat peritoneal macrophages (Ohuchi *et al.*, 1989) and potentiates the ability of TGF- $\beta$ 1 to upregulate uPA expression (Falcone *et al.*, 1993). The delayed upregulation of *c-fos*, *transin*, and urokinase by okadaic acid has also been demonstrated in mouse keratinocytes (Holladay *et al.*, 1992). It is likely that some proteases such as urokinase and collagenase are involved in angiogenic induction by okadaic acid because the expression of these two protease activities was induced by either okadaic acid or TPA (Holladay *et al.*, 1992; Kim *et al.*, 1990; Levy *et al.*, 1991; Whitham *et al.*, 1986).

### 8. Haptoglobin

Sera from patients with systemic vasculitis had the capacity to stimulate angiogenesis *in vitro* (Cid *et al.*, 1993), using the Matrigel model of angiogenesis. Haptoglobin was identified as one of the components of these sera able to mediate the angiogenic effect. Furthermore, antibodies to this protein partially inhibited the angiogenic activity of these sera. The angiogenic activity of haptoglobin was confirmed in two *in vivo* models: implanted disk and subcutaneous injection with Matrigel. This suggests that the increased levels of haptoglobin in chronic inflammatory conditions may play a role in tissue repair, and it may offset the effects of ischemia in systemic vasculitis by promoting the development of collateral vessels. Histopathological studies of affected tissues from systemic vasculitis patients often demonstrate new reparative vessels (Olsson *et al.*, 1990). It is not yet clear whether the enhanced angiogenic effect of haptoglobin from vasculitis sera is due to quantitative differences or to the presence of different haptoglobin with higher angiogenic activity.

### 9. Hyaluronic Acid Fragments

Fragments of hyaluronic acid between 4 and 25 disaccharides in length have been shown to be angiogenic in the corneal model (West *et al.*, 1985). Similarly sized hyaluronic acid fragments are also known to influence the binding to, and effect the interactions between, fibronectin and collagen (Yamada, 1981), and have been shown to cause aggregation of proteoglycans (Hascall and Heinegard, 1974) and self-association of the molecule to a considerable degree (Morris *et al.*, 1980). These events were similarly shown to be inhibited by the same sized hyaluronic acid fragments that inhibit angiogenesis (Eriksson *et al.*, 1983; Morris *et al.*, 1980). These findings support the idea that angiogenesis may be regulated at the level

of extracellular matrix, and that factors that influence its composition and integrity may influence the differentiation process at work in angiogenesis.

#### 10. Synergism between Gangliosides and Fibroblast Growth Factor

At suboptimal doses of angiogenic factors, the addition of gangliosides promoted angiogenesis (Ziches *et al.*, 1989). Molecules with a high sialic acid content, such as GT1b (bisialoganglioside), are more efficient at influencing the biological response of capillary endothelial cells than are molecules with lower sialic acids, such as GM1 (monosialoganglioside) (Alessandri *et al.*, 1986; Ziches *et al.*, 1989). Further studies show that gangliosides can synergize with bFGF and promote endothelial growth, motility, and survival (De Cristan *et al.*, 1990).

#### B. Inhibitors of Angiogenesis

As our knowledge of angiogenesis increases, so do the approaches used to inhibit this process. The existing inhibitors of angiogenesis target a variety of functions such as cell proliferation, migration, matrix-metabolizing mechanisms, matrix production, and cell-cell recognition.

##### 1. Fumagellin

The observation that a fungal contaminant in an endothelial cell culture produced an agent that perturbed the growth of the cells around the contaminant led Folkman *et al.* in conjunction with the Tekada Chemical Company to the isolation of fumagellin. Fumagellin inhibited angiogenesis in the CAM assay, but the levels required for prevention of solid tumor growth were cytotoxic. Synthesis of a more potent analog, *o*-(chloroacetylcarbomoyl) : fumagellol (AGM-1470), provided a safe and effective alternative with few side effects *in vivo*. However, although AGM-1470 inhibited endothelial proliferation *in vitro* it did not inhibit tumor cell growth. More recently another potent analog, TNP-470, did have the capacity to inhibit tumor growth *in vivo* (Ingber *et al.*, 1990; Kusaka *et al.*, 1994). The actions of TNP-470 appeared to be mediated through its ability to inhibit endothelial cell growth (Kusaka *et al.*, 1994). Current studies are directed toward generating a nontoxic analog effective in tumor regression.

##### 2. Inhibitors of Collagen Synthesis

Several reagents that modify the synthesis of collagen have been used to inhibit angiogenesis. The proline analog L-azetidine-2-carboxylic acid (LACA) prevents the triple-helical formation of collagen, and has been



shown to induce regression of growing capillaries in the CAM model (Ingber and Folkman, 1988) and inhibit branching or tortuosity in the rat mesenteric window model (Norrby, 1993). Another proline analog, *cis*-hydroxyproline, has been shown to block the synthesis and deposition of collagen in basement membrane and reduce the growth of rat mammary tumors (Lewko *et al.*, 1981; Wicha *et al.*, 1981). However, the ability to inhibit angiogenesis does not always correlate with the ability to act as an antitumor agent. Others have shown that LACA is ineffective as an antitumor agent (Klohs *et al.*, 1985). LACA cannot be hydroxylated and the newly synthesized polypeptides of procollagen do not fold into stable triple-helical conformations. When a critical number of prolyl residues have been substituted by the analog the thermal stability of the molecule is decreased, leading to alterations in the extracellular matrix, which is less able to support the normal proliferative capacity of the cell (Jimenez and Rosenbloom, 1974; Uitto and Prockop, 1974; Tay *et al.*, 1983). Titanocene dichloride, a reagent that inhibits the biosynthesis of collagens, has also been shown to be an active antitumor agent by suppressing angiogenesis (Bastaki *et al.*, 1994).

### 3. Protamine

Protamine, a 4.3-kDa arginine-rich protein, acts at a cellular site not associated with the FGF receptor, because although it inhibits the mitogenic effect of FGF it potentiates the mitogenic effect of epidermal growth factor (EGF) (Neufeld and Gospodarowicz, 1987; Majewski *et al.*, 1984). In addition, it has been shown to inhibit cross-linking between angiogenin and actin (Taylor and Folkman, 1982), an event directly related to the mechanism of action of this potent angiogenic factor. Protamine is not used for the control of neovascularization because of its unacceptable cytotoxicity (Folkman, 1985).

### 4. Cyclosporin A

The immunosuppressant drug cyclosporin A (CsA), administered as a long-term treatment during renal transplantation has been shown to have angiostatic properties in the rat mesenteric window model (Norrby, 1992). The mechanism of its angiostatic activity is not understood. However, a clue as to the mechanism of its actions may be derived from the demonstration that the CsA-sensitive transcription factor NFAT (nuclear factor of activated T cells) is present in endothelial cells (Cockerill *et al.*, 1994). The angiostatic effects of CsA may, therefore, be mediated through effects of the drug on genes regulated by the CsA-sensitive transcription factor NFAT.

### 5. Cartilage-Derived Factors

Extracts of cartilage, one of the few avascular tissues in the body, can inhibit angiogenesis (Eisenstein *et al.*, 1975; Brem and Folkman, 1975; Langer *et al.*, 1976, 1980). A protein, with sequence homology in the NH<sub>2</sub>-terminal region to collagenase inhibitor was purified from bovine scapular cartilage (Moses *et al.*, 1990). This protein inhibited proliferation and migration *in vitro* and angiogenesis *in vivo* in the CAM assay (Moses *et al.*, 1990). Because the dissolution of interstitial collagens is an important step in angiogenesis (Langer *et al.*, 1980; Rifkin *et al.*, 1982), the presence of collagenase inhibitors in cartilage explains its resistance to invasion and vascularization.

### 6. Heparin, Steroids, and Heparin-Steroid Conjugates

The control of angiogenesis with synthetic heparin substitutes was first demonstrated by Folkman and co-workers (1988). The angiostatic activity of heparin and nonanticoagulant heparin fragments was shown to be enhanced by administration of steroids (Crum *et al.*, 1985; Folkman and Ingber, 1987; Ingber *et al.*, 1986). Their mechanism of action was thought to be via induction of plasminogen activator inhibitor (PAI-1), thus affecting the breakdown of basement membrane (Blei *et al.*, 1993). The efficacy of these drugs was increased again by conjugating the two moieties. The covalent linking of a nonanticoagulating derivative of heparin (heparin adipic hydrazide) to antiangiogenic steroid (cortisol) via a labile bond generated a drug able to concentrate cortisol inside the vascular endothelium. The heparin moiety was able to target to the sulfated polyanion receptor on the cell surface, followed by endocytosis and release of cortisol inside the cell. The antiproliferative effect of these conjugates was far greater than that of cortisol and heparin administered in their unconjugated form (Thorpe *et al.*, 1993). The drugs were also shown to reduce vascularization of subcutaneous sponge implants and retard the growth of subcutaneous Lewis lung carcinoma by 65% (Thorpe *et al.*, 1993).

### 7. Platelet Factor 4

The platelet  $\alpha$ -granule protein PF4 was shown to inhibit angiogenesis (Taylor and Folkman, 1982), as was recombinant human PF4 (Maione *et al.*, 1990), and the CAM assay. Furthermore, PF4 completely suppressed the growth factor-dependent proliferation of human umbilical vein endothelial cells in culture (Maione *et al.*, 1990). Analysis of small peptides of the molecule suggests that the angiostatic activity was associated with the heparin-binding domain of the molecule, and addition of heparin in

experimental implants abrogated the effects of PF4. Platelet factor 4 has also been shown to have collagenase inhibitor activity (Hiti-Harper *et al.*, 1978).

### 8. Linomide

When given systemically to mice, linomide reduces primary and secondary tumor growth and metastasis of murine B16 melanoma cells (Kallard, 1986; Harning and Szalay, 1988; Passanti *et al.*, 1992; Vukanović *et al.*, 1993). The low toxicity of linomide, and its androgen-independent ability to inhibit tumor angiogenesis and hence suppress tumor growth, make it a putative clinically useful drug. Currently, its long-term effects are under investigation.

### 9. Placental RNase Inhibitors

Although RNase inhibitors are currently not feasible clinical reagents, as a result of their rapid clearance, they have significant antiangiogenic activities *in vitro*. It may be possible to conjugate these reagents with a protective protein to render them clinically useful. Placental RNasin binds to angiogenin hundreds of times more efficiently than it does to other ribonucleases (Fox and Riordan, 1990), suggesting a possible mechanism of action of this class of reagent. Studies using the corneal model and the subcutaneous implantation model demonstrate reduction of FGF- and orthovanadate-enhanced angiogenesis (Shapiro and Vallee, 1987; Polakowski *et al.*, 1993). RNasin prevented tumor growth of C755 mammary tumor cells. Furthermore, its antitumorigenic activity correlated with its effect on tumor-induced angiogenesis, suggesting that the ability of RNasin to inhibit tumor growth was due to its ability to inhibit angiogenesis (Polakowski *et al.*, 1993).

### 10. Hyaluronic Acid

Although hyaluronic acid (HA) fragments can be angiogenic, high molecular weight hyaluronic acid inhibits the vascularization of chick embryo limb bud (Feinberg and Beebe, 1983), and conversely the differentiation and vascular ingrowth are associated with an increase in tissue hyaluronidase activity (Toole, 1976; Belsky and Toole, 1983). Also, it has been shown that hyaluronic acid can reduce the rate of development of granulation tissue and newly formed capillaries around subcutaneous implants (Balazs and Darykiewicz, 1975). Studies by West and co-workers (1985) showed that the removal of HA may not only represent the removal of an inhibitor of angiogenesis, but that the degradative products of HA may be angiogenic.

### 11. Inhibitors of Oligosaccharide Processing

In a study of oligosaccharide processing, inhibitors of capillary formation in the fibronectin-induced model of capillary formation showed that the synthesis of hybrid-type oligosaccharides is required for capillary formation *in vitro*. During this process there is an increase in the synthesis of monosialated and fucosialated glycans on asparagine-linked oligosaccharides (Nguyen *et al.*, 1992). This observation may explain the mechanism whereby angiogenesis has been inhibited by antibodies directed against sialyl-Lewis-X and sialyl-Lewis-A (Lowe *et al.*, 1990; Phillips *et al.*, 1990; Walz *et al.*, 1990).

### 12. Tumor Suppressor Genes

Modulation of angiogenesis has been a possible function propounded for tumor suppressor genes (Bouck *et al.*, 1986; Sager, 1986). Demonstration that the expression of a 148-kDa protein in the culture medium of BHK 21/c113 (baby hamster kidney) cells was related to an active tumor suppressor gene, and that this protein inhibited angiogenesis in the corneal assay, supports this idea (Rastinejad *et al.*, 1989). The function of this gene is clearly not specific to hamster, as it can be complemented by chromosome 1 from normal human fibroblasts (Stoler and Bouck, 1985). The identity of the inhibitor is as yet unknown. Antibodies to the protein show no cross-reactivity to known antigens of this size. Weak cross-reactivity to collagen type IV was observed. However, the BHK inhibitor did not show the expected sensitivity to collagenase.

### 13. Thrombospondin

Good and co-workers were the first to identify thrombospondin (TSP) as being an inhibitor of angiogenesis (1990) when the amino acid sequence of an antiangiogenic tumor suppressor gene (Rastinejad *et al.*, 1989) was found to be similar to thrombospondin. Further studies showed that purified human TSP, isolated from platelets, was able to block neovascularization in the rat corneal model, and inhibits chemotaxis of capillary endothelial cells toward angiogenic factors (Good *et al.*, 1990). Its role as an angiogenic inhibitor was further supported by the elegant studies of O'Shea and Dixit (1988), who showed the presence of TSP to be adjacent to mature quiescent vessels, but absent from actively growing sprouts. This relationship was subsequently demonstrated *in vitro* (Iruela-Arispe *et al.*, 1991). In addition, the role of TSP as an angiogenic inhibitor is further supported by the inability of endothelial cells in fast-growing hemangiomas to make TSP (Sage and Bornstein, 1982), and the ability of antibodies to TSP to increase angiogenesis *in vitro* (Iruela-Arispe *et al.*, 1991). Throm-

thrombospondin mRNA has been shown to be downregulated in endothelial cells forming tubes in culture (Canfield *et al.*, 1986).

The mechanism of action of TSP is unclear, but is postulated to be related to modulation of adhesion interaction and growth because TSP can mediate cell-cell interactions, and may also play a role in cell-substrate interactions. For endothelial cells TSP can be adhesive. When endothelial cells are spread on other substrates the focal contacts can be broken by exposure to soluble TSP (Murphy-Ullrich and Hook, 1989; Murphy-Ullrich *et al.*, 1991). Thrombospondin has also been shown to inhibit endothelial cell growth (Bagavandoss and Wilks, 1990; Taraboletti *et al.*, 1990; Murphy-Ullrich *et al.*, 1992). Further studies showed that both the NH<sub>2</sub>-terminally truncated TSP, and a series of peptides from the procollagen-like region of the molecule, also blocked angiogenesis (Tolsma *et al.*, 1993). In more recent studies, TSP-containing fibrin and collagen matrices were able to promote angiogenesis in rat aortic explants on Matrigel (Nicosia and Tuszynski, 1994). These investigators showed that TSP directly stimulated the growth of aortic culture-derived myofibroblasts, which in turn promoted microvessel formation when cocultured with the aortic explants. This result is inconsistent with the interpretation of the *in vivo* studies, which show matrix-bound TSP in mature vessels, and report its absence in actively growing sprouts (O'Shea and Dixit, 1988).

#### 14. Estrogen Metabolites

The endogenous estrogen metabolite 2-methoxyestradiol inhibits angiogenesis and suppresses tumor growth (Fotsis *et al.*, 1994). This derivative is shown to inhibit cell proliferation and migration, and angiogenesis *in vitro*. It has also been shown to inhibit neovascularization and tumor growth in mice. This is the first steroid derivative to be active without heparin or sulfated cyclodextrins, indicating a different mechanism of action. 2-Methoxyestradiol has negligible interaction with the estrogen receptor (MacCluskey *et al.*, 1983). Although its mechanism of action is not fully understood, it has been shown to induce urokinase-type plasminogen activator, suggesting that modulation of endothelial cell proteolysis may be responsible, in part, for the inhibitory action of this compound (Fotsis *et al.*, 1993; 1994).

#### 15. Genistein

Genistein was isolated from the urine of vegetarians, and was shown to inhibit angiogenesis and cell proliferation (Fotsis *et al.*, 1993; Schweigerer *et al.*, 1992). This reagent was also shown to inhibit the production of plasminogen activator and plasminogen activator inhibitor in vascular

endothelial cells, suggesting a role in matrix metabolism. Genistein precursors are present in soy products, and its role as an angiogenic inhibitor correlates with the epidemiological data showing cultures consuming high soy diets (traditional Japanese) having a lower incidence of vascular tumors (Setchell and Adlercreutz, 1988; Adlercreutz *et al.*, 1991; Muir *et al.*, 1987).

#### 16. Synergism between Polysaccharides and Estrogen

Sulfated polysaccharide-peptidoglycan complex, isolated from *Athrobacter*, inhibited embryonic and tumor-induced angiogenesis and the growth of solid tumors (Inoue *et al.*, 1988). More recently, this reagent has been shown to synergize with Tamoxifen and  $\alpha$ -estrogen, and to reduce angiogenesis to a greater extent (Tanaku *et al.*, 1991).

#### 17. Angiostatin

The observation that some tumor masses were able to suppress tumor growth has recently led to the isolation of a 38-kDa inhibitor of angiogenesis, named angiostatin (O'Reilly *et al.*, 1994). This molecule is able to specifically inhibit endothelial cell proliferation, inhibit neovascularization, and the growth of metastases. Angiostatin shares considerable homology to an internal fragment of plasminogen, which corresponds to the first four Kringle regions of the molecule (Lerch *et al.*, 1990). The mechanism of its action is not yet known. It is interesting to note that angiostatin shares structural homology to hepatocyte growth factor (HGF), a glycoprotein suggested to act as a paracrine mediator of angiogenesis (Grant *et al.*, 1993). This raises the intriguing possibility that angiostatin could compete with HGF for its receptor, c-met (Tsarfaty *et al.*, 1992).

### IV. Concluding Remarks

The aim of further research must surely be to devise a more satisfactory regime of treatment to enhance angiogenesis where it would be beneficial, such as in wound healing, and to abrogate the process in solid tumors, where clearly their progress is dependent on the maintenance of a competent vascular supply.

Therapeutic modulation of angiogenesis is shown to be more effective through regimes that combine effective agents. To allow a more relevant evaluation of reagents with potential angiogenic responses it will be important to develop more sophisticated *in vitro* models that more closely parallel the *in vivo* situation. Searching for a single gene that determines this complex process is perhaps a simplistic and naive approach. It seems

more likely that greater advances are to be made in understanding the factors that influence those common molecules that we know are altered during angiogenesis. Understanding the factors that alter the extracellular milieu and alter gene expression during early events in angiogenesis will greatly assist the development of clinical regimes that modulate angiogenesis.

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COMMONWEALTH OF AUSTRALIA

*(Patents Act 1990)*

IN THE MATTER OF: Australian  
Patent Application 696764  
(73941/94). In the name of:  
Human Genome Sciences Inc.

- and -

IN THE MATTER OF: Opposition  
thereto by Ludwig Institute for Cancer  
Research, under Section 59 of the  
Patents Act.

Annexure GBC-10

This is **Annexure GBC-10** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
\_\_\_\_\_  
**Gary Baxter Cox**

WITNESS:

  
\_\_\_\_\_  
Patent Attorney

PEYTEE K. MCC

# CHAPTER 7

## ROLE OF CYTOKINES IN ENDOTHELIAL CELL FUNCTIONS

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**V**ascular endothelium, consisting of the cells and the extracellular matrices (ECM) that line the blood vessels, displays two characteristic properties: (a) endothelial renewal and angiogenesis, and (b) interactions with blood molecules and leukocytes. The turnover of endothelial cells (ECs) in most adult tissues is exceedingly slow, varying from just under 100 days in lung, liver, and mesentery to 1,000 days in brain, but replication periods may shorten 20 to 2,000 times during wound healing, inflammation, and malignancy, and are even faster in uteroplacental tissue and embryogenesis (1,2). Established endothelium regulates the blood coagulation system (3), the luminal diameter of its vessel (4), attachment of leukocytes to its surface and their migration into interstitium (5), and antigen presentation to lymphocytes (6).

Each of the endothelial functions described is coordinated by cytokines. These soluble, small molecular weight proteins are directed at ECs and their neighbors from adjoining mast cells, T cells, and macrophages, from other ECs, and from distant sites upstream in the circulation. Many cytokines possess binding sites on the ECM or endothelial surface, which prevent them from being washed away in the axial blood stream, potentially creating an important periendothelial cytokine reservoir (7,8). We discuss the cytokines involved in EC proliferation and interactions with the blood, with an emphasis on their contribution to pathophysiological mechanisms.

### RENEWAL AND ANGIOGENESIS

Endothelial renewal entails EC multiplication and migration (9,10). However, in addition to local EC division, there may be a role in vivo for a circulating pool of EC precursors, the existence of which has been implied by the finding of ECs in the bloodstream (11).

Angiogenesis, in contrast, summarizes a myriad of activities leading to the formation of an entire new blood vessel. It may be divided into component stages, using models such as cultured ECs on a three-dimensional (3D) gel or the growth of vessels on cornea or chorioallantoic membrane. New capillaries begin the process by sprouting from venules as ECs move in response to a migratory stimulus through basement membrane that has been locally degraded. The motile ECs elongate and align to create a solid sprout, which lengthens, canalizes, and joins other sprouts to form a loop. Pericytes adjoin the ECs, and the mature, stable capillary is formed (12). The place of cytokines in each stage may be studied.

### *Fibroblast growth factor*

**BACKGROUND:** Fibroblast growth factor (FGF) comprises a family of at least seven heparin binding growth factors sharing 35 to 55% amino acid identity.

FGF-1 and FGF-2 (acidic and basic FGF) are the best characterized isoforms and are the subject of this review, in which they are generally viewed synonymously. Each is a 154 amino acid polypeptide and together they are produced by most cell types, including ECs (13).

FGF is thought by some to be the principal cytokine involved in the *in vivo* induction of angiogenesis (9,14). It is expressed ubiquitously in normal adult tissues, but a paradox is raised in that endothelial proliferation in these tissues is generally quiescent, except in the female reproductive organs and at sites of pathology. This inactivity is not accounted for by FGF's lack of a classic signal sequence for secretion, because it is found extracellularly, possibly due to a truncated receptor, which serves as a secretory carrier (15). A more likely explanation is that the cellular responses to FGF are mediated by a receptor complex comprising heparin sulfate moieties, rather than by a single protein, which is subject to considerable polymorphism (16).

**EFFECTS ON ECS IN VITRO:** FGF stimulates proliferation and migration of cultured ECs (9,14,17), but, in addition to promitotic actions, it has several other effects that are likely to be important. One of the phenotypic hallmarks of migrating ECs is expression of plasminogen activator (PA), a central mediator of extracellular proteolysis. FGF up-regulates the synthesis of PA and collagenase (17-19). Wounding of an EC monolayer triggers a marked, rapid, and sustained increase in expression of a specific high-affinity receptor for the urokinase-type PA (u-PA) on the surface of migrating cells, the postulated role of which is to mediate efficient and spatially restricted extracellular proteolytic activity by migrating ECs (20,21). Increases in u-PA and u-PA receptor are both dependent on endogenous FGF.

EC invasiveness and formation of patent capillaries in fibrin and collagen gels is stimulated by FGF (22). It increases expression of the  $\alpha v \beta 3$  integrin, which correlates with an increased ability of microvascular ECs to bind to vitronectin, but not to fibronectin-coated surfaces (23). There is up-regulated biosynthesis of the collagen/laminin receptor, the  $\alpha 2 \beta 1$  integrin and the  $\alpha 5$ -chain, which conjugates with  $\beta 1$  to form a fibronectin receptor. These effects of FGF may provide ECs with an enhanced capacity to attach to, or migrate through, both their underlying basement membrane and the interstitial matrix (24).

**FGF INTRACELLULAR SIGNALING:** The signaling pathway of FGF appears to involve both a membrane receptor and a direct nuclear site of action. There are two FGF receptors, one of high affinity, which possesses an intracellular tyrosine kinase domain (25), and the other of low affinity, a heparan sulfate proteoglycan (HSPG) (26,27). It appears that binding to cell surface HSPG is a

prerequisite for high-affinity binding and mitogenic activity (16). Studies employing peptide mutants of FGF and anti-FGF antibodies to regulate the FGF-receptor interaction suggest that the mitogenic and PA-inducing activities of FGF depend on different domains of the FGF high-affinity receptor and different intracellular transduction pathways (28). Mitogenic activity involves triggering of protein kinase C (PKC), whereas plasminogen activation is independent of PKC, but requires a calcium flux (29).

FGF translocates to and accumulates in the nucleolus of ECs, independent of its high affinity receptor binding (30). It appears to stimulate the transcription of ribosomal genes during the transition from  $G_0$  to  $G_1$  phases of the cell cycle, a step closely linked to ribosome assembly and cell proliferation (31). Confluent ECs, in contrast to growing cells, contain no nuclear FGF (32). A mutant FGF lacking a nuclear translocation sequence fails to induce DNA synthesis and EC proliferation at concentrations sufficient to give rise to receptor-mediated tyrosine phosphorylation and *c-fos* expression (33).

A long-lasting interaction between FGF and cultured ECs with prolonged activation of PKC has been thought by others to be required to induce cell proliferation (34). Because high-affinity receptors lead to rapid internalization of FGF, and the low affinity sites mediate a slow internalization of FGF (35), the HSPG binding sites may be essential for FGF's growth factor activity.

**FGF-HEPARAN SULFATE PROTEOGLYCAN INTERACTIONS:** Heparan sulfate proteoglycans (HSPGs) are ubiquitous constituents of mammalian cell surfaces and most extracellular matrices. EC surface heparan sulfates facilitate the interaction of FGF with its receptor by concentrating FGF at the cell surface, possibly through phosphorylation of FGF at its receptor-binding domain because this process is associated with increased receptor affinity (16,36,37). FGF binding to HSPGs offers protection against proteolytic degradation and creates a reservoir of growth factor in tissues (8,38). Degradative enzymes may not be needed to release FGF from the heparan sulfates in instances where receptors and heparan sulfate-bound FGF are in close proximity because dissociation from heparan sulfates occurs rapidly enough to allow FGF to bind to unoccupied receptors by laws of mass action (36).

In a model of neuroepithelial embryogenesis that may hold clues for the endothelial scenario, Nurcombe and colleagues (39) showed that by sequentially binding different forms of FGF, differentially glycosylated HSPG species regulate development. This regulatory mechanism does not rely on changes in cell surface receptor expression or cessation of growth factor production and allows for rapid changes in cell signaling during development (39).



**FGF INHIBITORS:** The activities of FGF are limited by transforming growth factor- $\beta$  (TGF- $\beta$ ), interferon- $\gamma$  (IFN- $\gamma$ ), and platelet factor-4 (PF-4). TGF- $\beta$  can be activated from its latent, secreted form by plasmin, which is activated by FGF. TGF- $\beta$  then limits the PA-inducing activity of FGF through increased synthesis of PA inhibitor-1 (PAI-1) and decreased transcription of the u-PA gene (18). IFN- $\gamma$  inhibits EC growth possibly by decreasing FGF receptor expression (40). PF-4 blocks the binding of FGF to its receptor and therefore inhibits the migration and tube formation of bovine capillary ECs in culture (41).

A bacterially derived sulfated polysaccharide inhibits the growth and chemotaxis of ECs stimulated by FGF possibly by preventing the binding of FGF at both its low and high affinity binding sites (42). Chloroquine, a drug used to treat malaria and inflammatory diseases, inhibits FGF-stimulated EC growth in a dose-dependent fashion (43).

**EFFECTS IN VIVO:** Antisense oligonucleotides complementary to FGF messenger RNA (mRNA) illustrate the significant role of FGF as an endothelial growth promoter in an in vitro environment (44). In vivo experiments confirm that FGF has a critical part in the formation of new blood vessels.

Nanogram amounts of FGF induce angiogenesis in the chick embryo chorioallantoic membrane and in the cornea (45). Nabel and colleagues (46) used a eukaryotic expression vector encoding a secreted form of FGF-1 and introduced it by direct gene transfer into porcine arteries. In this somatic transgenic model, FGF-1 expression induced intimal thickening and angiogenesis within 21 days, in comparison with control arteries transfected with an *Escherichia coli*  $\beta$ -galactosidase gene. The neointimal ECs appeared to originate from adjacent arterial luminal ECs because both were negative for von Willebrand factor (46).

Using rabbit ear excision wounds and introducing various cytokines, Pierce and associates (47) showed that FGF induces an angiogenic response with a marked increase in ECs and neovessels. This effect appears to delay wound maturation. In contrast, platelet-derived growth factor (PDGF) augments early glycosaminoglycan and fibronectin deposition and induces greater amounts of collagen, whereas TGF- $\beta$ 1 rapidly enhances collagen synthesis and maturation. Each agent appears to have complementary actions (47).

Villaschi and Nicosia (48) found that addition of purified FGF increases both the number and the length of microvessels sprouting from the explants in a rat aortic injury model and prevents microvessel regression. Neutralizing anti-FGF antibodies cause a 40% reduction of angiogenesis (48).

### *Vascular endothelial growth factor/vascular permeability factor*

**BACKGROUND AND EC EFFECTS:** Vascular endothelial growth factor (VEGF) and vascular permeability factor (VPF) are two terms for an identical 46-kd protein related to PDGF and produced by several tumor cells, luteal cells, renal glomerular visceral epithelial cells, and vascular smooth muscle cells (VSMCs) (49–51). It is a selective and potent EC mitogen both in vitro and in vivo (50,52); it has no proliferative activity on VSMCs, fibroblasts, and epithelial cells (49). VEGF is angiogenic in vitro, and it causes microvascular ECs grown on 3D collagen gels to invade the underlying matrix and to form capillary-like tubules (52).

VEGF promotes vascular leakage, causes von Willebrand factor release, and synergizes with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) to promote procoagulant activity on ECs (49). In addition, it induces expression of the only metalloproteinase that can initiate the degradation of interstitial collagen types I to III under normal physiological conditions (53).

**VEGF SIGNALING:** Several tyrosine kinase receptors have been described for VEGF, including flt and flk-1 (2,54). These receptors are detected only on ECs. Flk-1 in particular is found on ECs during embryogenesis; it is especially abundant in blood islands of the yolk sac, where EC progenitors originate, and on vascular sprouts and branching vessels of developing brain. In contrast, flk-1 transcripts are drastically reduced in adult brain, in which vascular proliferation has ended (2). These findings contrast with the lack of detectable FGF receptors on ECs during embryogenesis (55).

VEGF binds heparin via a nonreceptor binding domain, which strongly potentiates its binding to flt, whereas  $\alpha$ 2-macroglobulin, a major serum protein, inactivates the receptor binding ability of VEGF (50). Akin to FGF, VEGF induces an angiogenic response via a direct effect on endothelial cells, and, when acting in concert, these two cytokines have a potent synergistic effect on the induction of angiogenesis in vitro (52).

**VEGF IN ANGIOGENESIS, MALIGNANCY, AND WOUND HEALING:** VEGF mRNA is expressed in cells surrounding an expanding vasculature in embryonic implantation sites, ovarian follicles, corpus luteum, and at sites of repair of endometrial vessels. It predominates in tissues that acquire a new capillary network, but its binding activity is found on both quiescent and proliferating ECs. VEGF expression may be hormonally regulated because it increases with the acquisition of cellular steroidogenic activity and varies with the ovarian cycle in the endometrium. During the early proliferative phase, it

is found in the estrogen-responsive, secretory columnar epithelium. Under the influence of progesterone in the secretory phase, when new blood vessel development is maximal, VEGF expression shifts to cells of the underlying stroma comprising the functional endometrium (56).

A particular role for VEGF in tumor angiogenesis is apparent. Expression of VEGF on Chinese hamster ovary (CHO) cells confers on them the ability to form tumors in nude mice (57). Monoclonal antibodies to VEGF inhibit the growth of rhabdomyosarcoma, glioblastoma multiforme, and leiomyosarcoma cell lines in nude mice, but they have no effect on the growth rate of these tumor cells in vitro, implying a direct effect on reducing the vascular density in antibody-treated tumors (57).

In situ analysis of glioblastoma multiforme brain tumor specimens shows that VEGF production is specifically induced in a subset of glioblastoma cells distinguished by their proximity to necrotic foci. Capillaries appear to cluster alongside these VEGF-producing tumor cells. VEGF mRNA levels are dramatically responsive to their  $O_2$  milieu, suggesting a mechanism for these findings. Within a few hours of exposing glioma and muscle cell cultures to hypoxia, VEGF mRNA levels increase and return to background when a normal  $O_2$  supply is restored (58). Comparison of astrocytomas with the more malignant glioblastoma, which is characterized by necrosis and vascular proliferation, reveals that more VEGF is expressed in the latter. Flt is not expressed in normal brain ECs, but it is found in these tumor ECs (59).

The other activity of VEGF on ECs—increased permeability—is a characteristic feature of normal wound healing. Persistent microvascular permeability to plasma proteins, even after cessation of injury, results in extravasation of fibrinogen, and the resultant fibrin serves as a provisional matrix that promotes angiogenesis and scar formation. VEGF mRNA levels in keratinocytes at wound edges are greatly increased, and they correlate with the permeability of wound tissue vessels (60).

#### *Hepatocyte growth factor/scatter factor*

Hepatocyte growth factor (HGF) and scatter factor (SF) are identical, basic, heparin-binding glycoproteins that share 38% amino acid identity with plasminogen (61,62). They are produced by fibroblasts and VSMCs. HGF induces renal epithelial cells to form branching networks of tubules in a collagen matrix (63), whereas SF disperses cohesive epithelial colonies and stimulates cell motility (64,65). The receptor for HGF/SF is c-met, a transmembrane tyrosine kinase (66), which is expressed and stimulated by HGF on ECs (67).

HGF stimulates EC growth and motility in vitro and promotes wound repair in EC monolayers. ECs assume a

dendritic phenotype. HGF does not induce procoagulant activity or platelet-activating factor (67), but EC secretion of plasminogen activators and urokinase, which are required during the early stages of angiogenesis when ECs degrade ECM, are induced by SF (68). In a corneal model, HGF produced angiogenesis (67). Immunoreactive SF is present in a perivascular distribution surrounding sites of blood vessel formation in the skin of psoriatic plaques, but not in normal skin, in which angiogenesis is not a feature (68).

#### *Transforming growth factor- $\beta$*

TGF- $\beta$  is unlike other cytokines with proangiogenic effects. Some investigators have found that TGF- $\beta$  induces tube formation by ECs in 3D collagen gels without affecting cell proliferation (69,70), but in other in vitro systems, it is a potent inhibitor of EC proliferation, migration, tube formation, and protease synthesis (71–75). Dramatic increases in collagen and fibronectin synthesis and inhibition of matrix-degrading enzyme production (76,77), brought about by TGF- $\beta$ , may modulate its effects on endothelial phenotype and explain differences between various in vitro models (69). In vivo TGF- $\beta$  is angiogenic, although it has been suspected that this action is not directly on ECs but rather on other cells (e.g., fibroblasts and macrophages, whose many products may potentiate the angiogenic response) (78,79).

TGF- $\beta$  may signal resolution of the angiogenic process begun by factors that are principally initiating stimuli, such as FGF and VEGF. Wound studies in vivo show that FGF produces marked increases in new vessels and persistence of the provisional ECM, whereas TGF- $\beta$  accelerates maturation of the provisional ECM through rapidly enhanced collagen synthesis, leading to coverage of wound defects with fibrous tissue (47,80). In chorioallantoic membranes, FGF induces primarily small blood vessels, whereas the vessels formed in response to TGF- $\beta$  are large and bear intercellular junctional complexes (70,79). TGF- $\beta$ 1 also promotes the differentiation of ECs into VSMCs in vitro (81) and stimulates intimal VSMCs to synthesize increased amounts of lipoprotein-binding proteoglycans (82).

TGF- $\beta$  is secreted constitutively by ECs as a high molecular weight inactive complex, with a latency-associated peptide and a latent TGF- $\beta$  binding protein. Activation of latent TGF- $\beta$  occurs in cocultures of ECs with VSMCs or pericytes and is thought to be at least in part mediated by plasmin cleavage of the aminoterminal propeptide. This process is facilitated by FGF, which increases plasminogen activator activity (18,72). Thus, the presence and effectiveness of TGF- $\beta$  are determined by proximal cell types and the ability to activate the latent form.

### *Hematopoietic colony-stimulating factors*

The colony-stimulating factors (CSFs) are characterized by their profound effects on the proliferation of blood cell precursors. ECs and hematopoietic cells share surface markers such as PECAM-1 (83,84), CD34 (85,86), CD36 (87), and CD45 (88), and they closely interact in the bone marrow, suggesting the common ancestry of their lineages and the possible effectiveness of the CSFs upon the former.

Granulocyte and granulocyte-macrophage CSFs (G-CSF, GM-CSF) have been shown to induce human ECs to migrate and proliferate, without altering their hemostatic or inflammatory phenotypes. In comparison to FGF, induction of migration was of a similar order of magnitude, although the extent of proliferation was less (89,90). In the corneal model, G-CSF induced neovascularization. An inability to repeat these results, however (91), and in particular the lack of demonstrable receptors for GM-CSF on human umbilical vein ECs (HUVECs) by binding analysis, surface expression, and receptor mRNA analysis (92,93), leave the significance of the original observations unclear.

In the last year, it has emerged that resting HUVECs express mRNA for the  $\alpha$ - and  $\beta$ -chains of the interleukin-3 (IL-3) receptor (90,91,94). This receptor is functional in mediating IL-3 stimulation of HUVEC DNA synthesis and proliferation (92). Furthermore, use of recombinant human IL-3 and GM-CSF after high-dose cancer chemotherapy is associated with expansion of the vascular network of the bone marrow and an increase in the proportion of CD34-expressing ECs (95). Erythropoietin is also reported to possess mitogenic and chemotactic activity on ECs (96).

### *Other cytokines affecting EC renewal and angiogenesis*

**ONCOSTATIN M:** Oncostatin M is a glycoprotein of 196 amino acids produced by activated T cells that inhibits the growth of several human tumor cell lines. It also inhibits the growth of normal bovine aortic ECs, while stimulating the growth of a number of fibroblast cell lines (97).

**INTERLEUKIN-4:** IL-4 is a potent EC mitogen (98), and it also stimulates the expression of u-PA (99), suggesting that it has a role in angiogenesis. Both these effects of IL-4 occur preferentially in microvascular, rather than in macrovascular, endothelium.

**INTERLEUKIN-8:** IL-8 has been reported to stimulate the chemotaxis of HUVECs with effects comparable to FGF. TNF- $\alpha$  or IL-8 antibodies reduce the chemotactic response for HUVECs to conditioned media from lipo-

polysaccharide-stimulated peripheral blood monocytes or synovial tissue macrophages. Antisense RNA oligonucleotide to IL-8 blocks the production of angiogenic activity by monocytes. IL-8 binds to heparin, as do the well characterized angiogenic factors, such as FGF and VEGF (100).

**EPIDERMAL GROWTH FACTOR:** Epidermal growth factor (EGF) is a mitogenic polypeptide that accelerates angiogenesis (101). EGF and EGF receptor immunoreactivity is present at the cytoplasmic interdigitations between ECs and pericytes in the angiogenic immature capillaries of human granulation tissue, but it is absent in mature capillaries (102).

**PLATELET-DERIVED GROWTH FACTOR:** PDGF is a mitogen and a chemotaxin for VSMCs and fibroblasts (103), and it has roles in wound healing and angiogenesis (47,104,105). It stimulates fibroblasts to synthesize collagen and collagenase, which leads to modification of the ECM; it is also a potent vasoconstrictor (101). PDGF is stored in platelet  $\alpha$ -granules, ECs, VSMCs, and macrophages, in particular those in newly formed atherosclerotic plaques (101,106). Two receptors,  $\alpha$  and  $\beta$ , are ligand-activated tyrosine kinases (107). IL-1, TNF- $\alpha$ , lipopolysaccharide, and blood flow induce accumulation of PDGF mRNA in ECs, whereas IFN- $\gamma$  and nitric oxide have the opposite effect (108,109).

**INSULIN-LIKE GROWTH FACTOR:** Insulin-like growth factor-1 is secreted by ECs from their basal abluminal surface. It supports VSMC and fibroblast growth in vitro (110,111).

**INTERLEUKIN-1:** IL-1 inhibits the proliferation of ECs in vitro, particularly when combined with FGF (112). It promotes growth of VSMCs and fibroblasts, possibly via increased PDGF release (106).

**ENDOTHELIN-1:** Endothelin-1 (ET-1) is a 21 amino acid peptide released from the endothelium. It elicits a variety of biological effects that include VSMC contraction and proliferation (113). ET-1 production by ECs is augmented by thrombin, G-CSF, TGF- $\beta$ , and IL-1 (114-116). Fluid shear stress induces rapid and significant down-regulation of ET-1 mRNA and peptide release (117).

### *Summary of the pathophysiological effects of endothelial mitogens*

Knowledge of the actions, structures, and encoding genes of the endothelial mitogenic factors has been applied to pathophysiology, and examples of the in vivo relevance of each cytokine have been given. A range of studies, includ-

ing those of cytokine or cytokine-induced mediator expression in pathological specimens and those of direct addition of a cytokine (e.g., by gene transfection) or its withdrawal (e.g., by antisense oligonucleotide), permit increasing levels of confidence with regard to conclusions of causality.

The descriptive method, which is more suited to human samples, is exemplified by work on VEGF expression in the uterus (56) and the role of angiogenic factors in rheumatoid arthritis. Synovial pannus tissue in rheumatoid arthritic joints is invasive and destructive of adjacent cartilage and bone. Its formation is accompanied by ingrowth of new vascular networks, and the level of angiogenic activity correlates with infiltration of inflammatory cells, synovial hyperplasia, and clinical score (118,119). FGF expression has been shown in ECs of the rheumatoid pannus and in streptococcal-induced arthritis in rats (120,121). EGF and PDGF localization is also associated with areas of new vessel growth (122). Agents effective in the therapy of rheumatoid arthritis inhibit EC proliferation (123,124).

A study of injured rat aorta implicates FGF more conclusively in endothelial cell responses, but its application in human disease has not been established. Immunohistochemical staining of rat aorta shows FGF in the cytoplasm of endothelial and smooth muscle cells. Endogenous FGF is demonstrable by Western blot analysis in aorta-conditioned medium after ring dissection. Neutralizing anti-FGF antibodies inhibit the increased numbers and length of sprouting microvessels provoked by injury, suggesting that the FGF present is functional. Purified FGF increases both the number and the length of microvessels sprouting from the explants, particularly late after the injury, when release of endogenous FGF is minimal. FGF release by vascular cells thus appears to have a role in the autoregulation of angiogenesis after vascular injury (48).

Surgical interventions, such as bypass grafting, atherectomy, or endarterectomy, involve similar vascular injury, but they are also often complicated by intimal hyperplasia of VSMCs. The increased rate of migration and turnover of VSMCs in response to injury is dependent on the presence of dividing ECs. VSMC proliferation is maximal when the ECs are proliferating. When the ECs stabilize and cover the luminal surface, VSMC proliferation slows. The relationship of this phenomenon to EC-derived FGF or PDGF is not clear (125), although the failure of confluent ECs to localize FGF to their nuclei is interesting (32). TGF- $\beta$  also decreases VSMC proliferation (72). This effect may explain why VSMC proliferation slows when ECs cover an injury.

A recent innovation of *in vitro* assays—coculturing ECs with their supporting stromal cells to parallel the *in vivo* situation—provides another demonstration of interactions between ECs with their neighboring pericytes or VSMCs. Pericytes grown in coculture with ECs inhibit EC

proliferation (126), on the basis of the ability of cocultures but not homocellular cultures to produce activated TGF- $\beta$  (72). Angiogenesis in the fetal retina ceases as mural pericytes appear, and pathological neovascularization in diabetic retinopathy is associated with loss of pericytes (127).

EC cocultures with keratinocytes shed light on the common dermatological disease psoriasis, which is characterized by hyperproliferation of keratinocytes and abnormally extensive dermal capillary networks. Keratinocytes produce TGF- $\alpha$ , which in an autocrine manner leads to their hyperproliferation. TGF- $\alpha$  also stimulates human omental microvascular ECs in type I collagen gels to form tubular-like structures. When keratinocytes are cocultured with omental ECs, tubular-like EC structures appear in collagen gels, which are inhibitable by anti-TGF- $\alpha$  antibodies (128). It thus appears that TGF- $\alpha$  acts in an autocrine fashion on keratinocytes and in a paracrine manner on ECs, therefore appearing to facilitate the neovascularization required to allow for the increased surface keratinocyte population.

Angiogenesis appears to be closely related to malignancy (129). Angiogenic agents have been isolated from tumors, and neovascularization is present in most malignancies at the time of their detection, thus appearing to be directly related to malignant grade and prognosis. The malignant progression of melanoma from normal skin to dysplastic melanocytic nevus, to cutaneous malignant melanoma, and finally to metastatic malignant melanoma is associated with increasing vascularity (130). Horak and associates (131) showed that the number of microvessels in primary breast cancers is directly correlated with pathological indicators of an increasingly poor prognosis. The association of VEGF with rhabdomyosarcoma, glioblastoma multiforme, and leiomyosarcoma is an example of the role of cytokine-induced angiogenesis in malignancy (57). Tumor-cell hypoxia is believed to stimulate production of VEGF. In acquired immunodeficiency syndrome-related Kaposi's sarcoma, the malignant cells also express cytokines with autocrine and paracrine growth effects, which foster growth of this vascular tumor (132–134). Coculture of ECs with human glioblastoma cells demonstrates how angiogenic cytokines may be working. In contact with glioma cells, ECs form tubes, an *in vitro* surrogate for capillaries. Only glioma-cell lines that possess high levels of FGF mRNA induce such tube formation, and this process can be blocked by coadministration of anti-FGF antibody (135).

Because EC renewal and angiogenesis are key events in important pathological processes such as wound healing, inflammation, and malignancy, but are not a feature of normal tissues except in the female reproductive system, attempts to define angiogenic inhibitors have been in progress. Physiological inhibitors of angiogenesis are present in blood and urine, and they include cortisol me-

tabolites that lack glucocorticoid and mineralocorticoid activities (136). These metabolites may act by increasing the synthesis of PAI-1 (137). Heparin potentiates their action, and it is believed to function by virtue of its high affinity for angiogenic factors, such as FGF and VEGF (138).

IFN- $\alpha$  has been used successfully in pulmonary hemangiomatosis with an associated decrease in the density of abnormal vessels (139). Derivatives of *Aspergillus fumigatus* products are effective inhibitors of angiogenesis in vitro, as well as in tumors and collagen-induced arthritis in rats, with minimal toxicity (140,141). A number of other compounds inhibit angiogenesis in vitro, including antiestrogens (142), cyclosporine (143), ribonuclease inhibitors (144), protein kinase inhibitors (145), and calcium channel blockers (146). In vitro experimentation has shown that changes in the constitution of ECM or monoclonal antibodies directed to  $\beta 1$  and  $\beta 3$  integrins, which mediate EC-matrix interactions, can alter the type and number of capillary tubes formed (147,148). This finding offers prospects for the development of targeted inhibitors of angiogenesis.

### ENDOTHELIAL CELL INTERACTIONS WITH BLOOD MOLECULES AND LEUKOCYTES

In this section, the roles of cytokines acting on or produced by endothelia, which affect its relationship with the elements of blood, are considered. It appears that specific, separate mechanisms exist that deal with the arrest of mobile leukocytes, their secure attachment to the endothelial surface, and finally their passage between surface ECs into the interstitium. These inflammatory processes and others leading to thrombosis or immune recognition are controlled by specific cytokines with both facilitatory and inhibitory actions.

#### *Tumor necrosis factor- $\alpha$ and interleukin-1*

TNF- $\alpha$  and IL-1 are proinflammatory cytokines with effects on multiple biological systems. Both act on ECs, most often with a similar outcome; thus, they are considered together, but mention will be made of each individually when their effects differ.

IL-1 is a 17-kd peptide with two biologically active forms, IL-1 $\alpha$  and IL-1 $\beta$  (149). TNF- $\alpha$  is a trimer of three 17-kd subunits. Its main source is the monocyte-macrophage when it is stimulated by lipopolysaccharide (LPS), IL-1, IFN- $\alpha$ , IFN- $\gamma$ , OR GM-CSF (150). The mast cell may be another important source of TNF- $\alpha$  in vivo (151). Peripheral blood lymphocytes, natural killer (NK) cells, and

polymorphs produce a relatively small amount of TNF- $\alpha$  (150). VSMCs adjacent to the endothelium express TNF- $\alpha$  mRNA and protein on exposure to inflammatory signals, and this process is superinduced by protein synthesis inhibitors (152). Sources of IL-1 are extensive and include blood monocytes, neutrophils, T and B lymphocytes, tissue macrophages, VSMCs, and ECs. The most common stimulus for IL-1 transcription is endotoxin (149).

In general, TNF- $\alpha$  promotes selective cytotoxicity and catabolism. There are changes in ECM through induction of collagenases, which lead to bone resorption, and of plasminogen activator, which leads to angiogenesis (153). On blood cells, TNF- $\alpha$  and IL-1 have a proinflammatory effect. Neutrophils and monocytes gain an increased capacity for phagocytosis, antibody-dependent cellular cytotoxicity, degranulation, and production of reactive oxygen species. T cells express more IL-2 receptor and major histocompatibility complex (MHC) class II antigens. They achieve a greater proliferative potential in synergy with IL-2, and they produce more IFN- $\gamma$ . B cells also augment their proliferation, differentiation, and production of antibodies (149,150). The general proinflammatory effects of these cytokines, however, also depend on their endothelial functions, as well as on these leukocyte changes.

**COAGULATION:** TNF- $\alpha$  and IL-1 regulate the coagulation system through actions on the endothelium. Procoagulant activity of ECs is induced (3,154). The production of thrombomodulin, a surface glycoprotein on ECs that controls intravascular coagulation cascades through interactions with proteins C and S, is suppressed (154,155). Arachidonate metabolism is activated; in particular, prostacyclin synthesis in cultured vascular ECs is induced (156). TNF- $\alpha$  induces expression of an EC plasminogen activator inhibitor (157-159). Effects on plasminogen activator are recognized, but both increases and decreases have been reported (153,157). In patients treated with recombinant human TNF- $\alpha$  in a Phase I cancer trial, induction of endothelial-derived tissue-type plasminogen activator, recognized by the presence of fibrin degradation products in plasma, occurred within one hour of the initiation of TNF- $\alpha$  (160).

The surface of TNF- $\alpha$ -activated ECs elicits a hemostatic response when exposed to flowing nonanticoagulated blood. Tissue factor is expressed, and deposition of fibrin, platelet aggregates, and leukocytes follows; in an experimental model, 63% of the EC surface became covered. Resting ECs, in contrast, show no or little fibrin, platelet, and leukocyte deposition. The addition of antibodies against tissue factor to TNF- $\alpha$ -activated ECs abrogates fibrin and platelet deposition, but it allows leukocyte adherence to occur to the same extent. Thus, the endothelial effects of TNF- $\alpha$  on hemostasis and leukocyte

capture involve related but dissociated mechanisms (161).

**ADHESION:** TNF- $\alpha$  and IL-1 increase the adhesiveness of endothelium for bloodstream leukocytes by an EC-specific mechanism (162). They also modulate the interactions between adjoining ECs. This process involves induction, up-regulated expression, or activation of molecules on the endothelial surface, which interact with ligands on leukocytes and ECs. The main groups of such regulated adhesins are the selectins, the L-selectin ligands, the immunoglobulin superfamily, and the integrins.

**SELECTINS:** E-selectin (ES; ELAM-1) and P-selectin (PS; GMP-140; CD62) are endothelial, lectin-bearing glycoproteins that mediate the initial contacts between leukocyte and endothelium as leukocytes move away from the axial bloodstream and roll over the endothelial surface, prior to their firm attachment (163–165). Their leukocyte ligands are sialylated, fucosylated oligosaccharides (166).

ES is found in low to undetectable quantities on resting ECs *in vitro* and *in vivo*. Both IL-1 and TNF- $\alpha$  induce ES mRNA and protein synthesis. Surface expression *in vitro* reaches a maximal level at 4 hours, after which it declines to background by 24 hours, despite the continued presence of the agonists (167–170). Mast-cell degranulation leads to the display of ES on adjacent ECs *in vivo*. Antiserum to TNF- $\alpha$  abrogates this potentially important pathway of induction (151). Surface ES is lost by internalization and external release, processes not known to be controlled by cytokines (171). The rate of internalization of ES is comparatively higher than that of intercellular adhesion molecule (ICAM)-1; therefore, its pro-adhesive effects may be relatively acute (1.7% of membrane-bound ES/min vs < 0.1% of membrane-bound ICAM-1/minute) (172,173).

PS is expressed in varying levels on resting ECs, which maintain a large stock of PS in cytoplasmic granules (Weibel-Palade bodies). This store is rapidly transferred to the EC membrane in response to histamine and thrombin, but expression is brief, and its functional aspect is lost within 30 minutes (174,175). PS is inducible by TNF in mouse and bovine ECs at both mRNA and cell surface protein levels. The PS protein increase is 2- to 4-fold, and it is maximal at 3 to 4 hours (176).

**L-SELECTIN LIGANDS:** CD34 is a highly glycosylated, negatively charged, sialomucin-like transmembrane molecule better known for its presence on hematopoietic progenitor cells, which has recently been recognized as an endothelial ligand for L-selectin (LS; LAM-1; LECAM-1), the leukocyte adhesion molecule (177,178). It has a broad EC distribution, including high endothelial venules, but it is absent from most large vessels and placental sinuses (178). The function of CD34 is likely to be con-

trolled by differential vessel-specific glycosylation, as well as translocation to and oligomerization at the EC surface. Vessel-specific glycosylation may explain why leukocytes only adhere to postcapillary venules, even though CD34 appears to be expressed on capillaries and venules (162). TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$  have been reported to decrease the expression of CD34 on cultured ECs (179).

Glycam-1 (or Sgp50) is a 50-kd, mucin-like glycoprotein that also serves as an LS ligand. It is restricted, however, to lymph node high endothelial venules, and it is not known to be influenced by cytokines (180). Both CD34 and Glycam-1 were characterized by precipitation with a chimeric molecule containing the extracellular domain of mouse LS and human immunoglobulin.

A third endothelial ligand for LS must be proposed on the basis of older evidence describing an antigen on HUVECs that is optimally induced by TNF- $\alpha$  and IL-1 $\beta$ , but also to a lesser extent by IFN- $\gamma$  and IL-4 (181). This ligand is a neuraminidase-sensitive molecule, which implies that it bears sialic acid, as do CD34 and Glycam-1. It is expressed between 2 and 4 hours after HUVEC stimulation, and it persists for at least 24 hours (181). Its induction by cytokines is thus unlike CD34, and its distribution is wider than that known for Glycam-1.

Soluble LS is shed from the surface of leukocytes after their stimulation by cytokines such as TNF- $\alpha$ . Soluble LS inhibits LS-specific attachment of lymphocytes to TNF- $\alpha$ -activated ECs (182). Fluid-phase PS also inhibits adhesion of TNF- $\alpha$ -activated neutrophils to resting ECs, through inhibition of a CD18-dependent process. The control of soluble PS production is not currently understood (183).

**IMMUNOGLOBULIN SUPERFAMILY ENDOTHELIAL ADHESINS:** Intercellular adhesion molecule-1 and -2 (ICAM-1, 2), vascular cell adhesion molecule-1 (VCAM-1), and platelet endothelial cell adhesion molecule-1 (PECAM-1; CD31) are members of the immunoglobulin superfamily. ICAM-1, and ICAM-2 and VCAM-1 interact with leukocyte  $\beta$ 2 and  $\beta$ 1 integrins respectively, to serve a shear-resistant adhesion between ECs and leukocytes, which follows and stabilizes the rolling attachment initiated by the selectins (148). ICAM-1, the ligand for CD11a-CD11b/CD18, is constitutively expressed on ECs. TNF- $\alpha$  or IL-1 increase its expression, with a plateau at 24 to 72 hours (169). ICAM-2 is also constitutively expressed on ECs, but it is not subject to regulation (184).

VCAM-1 expression is absent on resting ECs. TNF- $\alpha$  or IL-1 induce it within 2 hours (peak, 12–24 hours), and levels substantially higher than baseline persist for at least 72 hours (185,186). There are two isoforms of VCAM-1 with 6 and 7 Ig-like domains and subtly different binding characteristics (187). The former is expressed earlier than the latter, which then becomes the main EC isoform. VSMCs are normally devoid of adhesion proteins *in vivo*, and they express only small amounts of VCAM-1 in cul-



ture. TNF- $\alpha$  and IL-1 induce human saphenous vein SMCs to express VCAM-1 (Gamble JR, et al., unpublished observations).

PECAM-1 is constitutively expressed in a homogeneous pattern on EC membranes *in vivo*, but it concentrates predominately at points of cell-to-cell contact on cultured ECs (188,189). It appears to have a role in EC-to-EC contacts and in leukocyte transmigration through the intercellular junctions of ECs (190). Using tissue specimens from breast, skin, stomach, colon, uterine cervix, endometrium, myometrium, and bronchus incubated with TNF- $\alpha$  for up to 6 hours, as well as a dermal ragweed antigen injection model, Ioffreda and colleagues (191) showed that TNF- $\alpha$  leads to a redistribution of PECAM-1 from its original uniform pattern on EC surfaces to one localized to areas of contact between adjacent ECs (191). Thus, TNF- $\alpha$ -stimulated molecules, ES and PECAM-1, may sequentially enhance leukocyte-EC binding in postcapillary venules, direct adherent cells to sites most conducive to transvascular diapedesis, and lead to transmigration.

**INTEGRINS:** ECs express a number of integrins believed to function as interendothelial adhesins or as ECM receptors. TNF- $\alpha$  and IFN- $\gamma$  decrease EC  $\beta$ 3 integrins, and they induce  $\alpha$ 1 $\beta$ 1. The  $\alpha$ 1 $\beta$ 1 integrin is normally absent from HUVECs, but is present on capillary ECs. The laminin receptor  $\alpha$ 6 $\beta$ 1 is strongly decreased by TNF- $\alpha$  or IL-1 $\beta$ , whereas  $\alpha$ 2 $\beta$ 1,  $\alpha$ 3 $\beta$ 1, and  $\alpha$ 5 $\beta$ 1 are not altered. Laminin adhesion by ECs is consequently decreased (192).

**MAJOR HISTOCOMPATIBILITY COMPLEX GENES:** Class I MHC is expressed on ECs, but at a level relatively lower than that on macrophages or lymphocytes. This expression is increased 2- to 4-fold over 24 hours by TNF- $\alpha$  (but not by IL-1) through an increase in transcription. There is a synergistic elevation in class I antigen with IFN- $\gamma$  or IFN- $\beta$ , without any alteration in TNF receptor number due to a multiplicative increase in transcriptional rate (193,194). Synthesis and expression of transporter in antigen processing-1 (TAP-1), an MHC-encoded gene product that is required for efficient association of intracellular peptide antigen with nascent human leukocyte antigen (HLA) class I H-chain and  $\beta$ 2-microglobulin, are increased in human ECs by TNF- $\alpha$ , IFN- $\beta$ , or IFN- $\gamma$  (195). TNF- $\alpha$  does not induce class II expression *de novo* in human ECs (193), and neither TNF- $\alpha$  nor IL-1 alters MHC on VSMCs (196). TNF- $\alpha$  thus allows ECs to present class I-restricted antigens to T cells, which may propagate the inflammatory response.

**ENDOTHELIAL PERMEABILITY TO MACROMOLECULES:** The permeability of endothelial surfaces to macromolecules such as albumin is an important feature of edematous processes. TNF- $\alpha$ , IL-1, and IFN- $\gamma$  increase EC monolayer permeability to albumin *in vitro*,

and this effect is augmented by combining these cytokines (197,198). The ECs undergo parallel changes in morphology, from cobblestone to elongated cells, with formation of prominent intercellular gaps and actin stress fibers. There is an accompanying loss of fibronectin and remodeling of the ECM (199). ECs from different sites show varying susceptibility to this change: HUVECs require prolonged exposures of 72 hours, whereas bovine ECs demonstrate a change within 1 to 3 hours after exposure (198,200).

Endothelial glycosaminoglycans (GAGs) are important in regulating vascular permeability as well as cell interactions with soluble factors and resistance to thrombosis. IL-1 and TNF- $\alpha$  alter GAG metabolism in cultured HUVECs, causing a marked increase in culture supernatant GAGs and a decrease in cell-associated GAGs that is detectable after 12 to 48 hours of coincubation. There is a concomitant increase in GAG synthesis. Histochemically, these changes are associated with marked reduction and redistribution of endothelial surface anionic sites. Such changes may contribute to the disturbances of vascular endothelial homeostasis associated with inflammatory states (201).

**LEUKOCYTE TRANSMIGRATION:** HUVECs incubated with TNF- $\alpha$  or IL-1 for 4 hours do not display morphological changes or increased albumin permeability, but they do show an increase in leukocyte transmigration (202). Migration occurs across EC junctions, and it is dependent on the ECs being biosynthetically active. Neutrophils are polarized but not degranulated, and there are no signs of ECM lysis. Because the number of traversing neutrophils is no greater when cytokine stimulation is joined by a chemotactic gradient in some systems, it is suggested that both operate through this mechanism (203-205).

Conditioned media of TNF- $\alpha$ - or IL-1-stimulated ECs induce transmigration of neutrophils when added to the basal EC compartment. IFN- $\gamma$ , IL-2, PDGF, and platelet-activating factor (PAF) are unable to mimic this effect. Antisera to IL-6, G-CSF, and GM-CSF, all products of stimulated ECs, do not diminish the chemotactic activity of the conditioned medium. IL-8, a member of the chemokine family, is present in this conditioned medium (206), and it acts as a chemoattractant for granulocytes. Other products of activated ECs, such as macrophage inflammatory protein-1 (MIP-1) and monocyte chemotactic protein-1 (MCP-1), are selective for lymphocyte and monocyte chemotaxis. Adhesion to ECs and transmigration through EC monolayers by leukocytes are thus both facilitated by TNF- $\alpha$  or IL-1 in separate, sequential processes (204).

**EFFECTS ON CYTOKINE PRODUCTION:** TNF- $\alpha$  and IL-1 foster EC production of a number of cytokines that often have proinflammatory actions (e.g., IL-1 itself)

(106,207), the chemokines (207), and G-CSF and GM-CSF (208,209). They mediate the release of PDGF from cultured ECs, and they augment IL-6 secretion (106, 210–212). IL-1 increases EC production of ET-1 (116).

Synthesis and release of the signaling phospholipid PAF is stimulated by TNF- $\alpha$  or IL-1 $\alpha$  treatment of ECs (213). Such ECs support adhesion of neutrophils that are unactivated and do not adhere to plastic, suggesting that PAF may be a proadhesion signal from ECs to neutrophils. Although PAF antagonists inhibit adhesion, the time course for adhesion and PAF production are not strictly concordant. Acetyl coenzyme A raises PAF levels, but it has no effect on adhesion, and although both IL-1 $\alpha$  and IL-1 $\beta$  stimulate adhesion, only the former results in PAF production (214).

TNF- $\alpha$  also decreases expression of endothelial antigens. Westphal and associates (215) reported a monoclonal antibody recognizing a 180-kd molecule expressed on the EC luminal surface, which is down-regulated by TNF- $\alpha$  and is possibly endoglin, a TGF- $\beta$ -binding cell surface protein.

**EFFECTS ON LIPOPROTEIN METABOLISM:** TNF- $\alpha$  or IL-1 increase low density lipoprotein (LDL) receptor expression on microvascular ECs in culture. There is a parallel increase in internalization and degradation of LDL (216). Because ECs oxidize LDLs, and because LDLs are atherogenic, cytokines produced by adherent monocytes found in early atherosclerosis may facilitate this pathological process.

### INTRACELLULAR SIGNALING

**TNF- $\alpha$  RECEPTORS:** TNF- $\alpha$  acts on cells via two receptors, p55 and p75, which are partially homologous in their extracellular domains, but lack any intracytoplasmic similarity. The role of these two moieties is still controversial, and it varies with the analytical method (217). ECs possess both receptors (218). Using TNF- $\alpha$  mutants with preferential binding to either p55 or p75, Barbara and colleagues (219) showed that the p55 receptor is necessary for induction of ES, neutrophil transmigration across EC monolayers, and EC IL-8 secretion. The p75 receptor only facilitates an increase in the potency of TNF (219,220). N-terminal amino acids of TNF- $\alpha$  are also critical for both receptor binding and biological activity on ECs (221).

**POSTRECEPTOR PATHWAYS:** TNF- $\alpha$  increases EC monolayer permeability via a G-protein intermediary (200), but activators of the stimulatory or inhibitory guanine nucleotide-dependent binding proteins do not affect TNF- $\alpha$ -induced surface expression of ES or VCAM-1 (222).

There is partial evidence of protein kinase C and A (PKC, PKA) involvement in the induction of ES expres-

sion and IL-6 production. PMA and forskolin, both agonists of these respective protein kinases, can mediate these effects, and appropriate kinase inhibitors impede them. These kinase inhibitors, however, do not block the effects of TNF- $\alpha$ , and other cyclic adenosine monophosphate agonists are not effective (193,222).

Absence of PKC translocation from cytosol to the plasma or nuclear membrane particulate fractions of HUVECs after TNF- $\alpha$  exposure, has argued against a significant PKC-mediated pathway for the actions of TNF- $\alpha$ . The  $\beta$ -I PKC isozyme, however, becomes activated without translocation, and it is sufficient for expression of ES and VCAM-1. This evidence from Harlan's group (223) suggests that PKC may mediate some effects of TNF- $\alpha$ . PKC is also strongly implicated in TNF- $\alpha$  induction of tissue plasminogen activator, because this substance is interdicted by the PKC inhibitors, H7 and staurosporine, and it is stimulated by 4 $\beta$  phorbol, 12 myristate, 13-acetate (PMAs) (153).

IL-1 $\beta$ -mediated endothelial cell phospholipase A2 activity and prostacyclin synthesis occur via a novel transducing pathway that does not involve early activation of phospholipase C, phospholipase D, or adenylate cyclase (224).

**ACTIVATION OF TRANSCRIPTION FACTORS:** TNF- $\alpha$  and IL-1 signaling on ECs involves activation of the transcription factors AP-1, NF-kappa B (NF-kB), interferon regulatory factor 1, cAMP response element (CRE), and TRE (PMA response element) (193,225). This level of the signaling pathway offers some explanation for the selective endothelial induction of ES. Two proximal ES promoter elements, in addition to NF-kB, are essential for cytokine induction of ES transcription. One of these elements, however, is not endothelial-specific, because it can function as a T-cell enhancer, as well as cooperate with NF-kB to yield cytokine induction of ES gene transcription in ECs (226). DNA methylation of the ES promoter represses NF-kB transactivation in nonendothelial cells, and, in comparison, the ES promoter in ECs is undermethylated, suggesting that methylation could have a role in cell-type-specific expression of this gene (227).

The cytokine-responsive regions of the VCAM-1 promoter are functional NF-kB and GATA elements (228). A comparison of the transcriptional control of VCAM-1 in muscle and ECs is enlightening. Muscle cells display high basal VCAM-1 expression that is not cytokine-inducible; a position-specific enhancer overrides other promoter elements. ECs have octamer binding sites that act as silencers, thus dampening VCAM-1 expression in unstimulated cells. TNF- $\alpha$  overcomes this inhibition through two adjacent NF-kB sites (229).

NF-kB induction by IL-1 $\alpha$ , TNF- $\alpha$ , and LPS is inhibited by I-kappa B $\alpha$  (IKB $\alpha$  or MAD-3), which sequesters NF-kB to the cytoplasm. Cell stimuli, such as TNF- $\alpha$  or



PMA, cause rapid degradation of IKB $\alpha$ , thus relieving this inhibition and allowing NF-kB to translocate to the nucleus and transactivate its target genes. Following this process, there is a dramatic increase in IKB $\alpha$  mRNA and protein synthesis. Expression of IKB $\alpha$  is also inversely regulated by NF-kB in a negative-feedback mechanism: NF-kB down-regulates its own activity after transient activation of target genes has been achieved (230,231).

## DISEASE ASSOCIATIONS

**ENDOTHELIAL DAMAGE:** Several diseases are associated with elevated serum or tissue levels of TNF- $\alpha$  or IL-1, such as idiopathic pulmonary fibrosis (232), systemic vasculitis (233,234), rheumatoid arthritis (235), psoriasis (236), cerebral malaria (237), and sepsis (238). All these disorders have some form of vascular pathology.

There is *ex vivo* evidence that supports the role of TNF- $\alpha$  and IL-1 in EC injury in Kawasaki's disease (KS). KS histopathology shows panvasculitis with endothelial necrosis and Ig deposition. Sudden death stemming from coronary arteritis is well recognized in this condition. Circulating antibodies in patients with KS display complement-dependent cytotoxic activity against IL-1 or TNF- $\alpha$ -inducible EC antigens, but not against resting ECs (239).

*In vitro* models of endothelial injury also suggest that these cytokines may bring about EC damage. After IL-1 activation, for instance, EC monolayers coincubated with unstimulated neutrophils show extensive EC detachment and loss of monolayer integrity. This process is mimicked by neutrophil elastase exposure, and it is prevented by serine protease inhibitors or avoidance of direct EC-neutrophil contact (240).

**ADHESION MOLECULE EXPRESSION IN TISSUES:** Examination of the tissue expression of adhesion molecules confirms their association with inflammatory diseases, and it indirectly implies that TNF- $\alpha$  and IL-1 exhibit widespread endothelial activity.

Normal peripheral lymph node and mucosa-associated lymphoid endothelium show no VCAM-1, but they do exhibit ICAM-1. VCAM-1 is present in follicular centers and interfollicular zones. ECs in most other normal tissues express little or no VCAM-1, but focal reactivity is seen in arterial vasa vasorum, hepatic Kupffer's cells, and some renal tubular epithelial cells (241). ES is absent from normal capillaries, but it is found on large vessel and umbilical vein endothelium (242).

In acute appendicitis or diverticulitis, strong VCAM-1 and ES staining is seen in ECs of dilated serosal venules. Lymphadenitis (sarcoid or toxoplasmal) shows focal venular VCAM-1, but there is little or no ES. VCAM-1 staining is stronger and more widespread in cat-scratch lymphadenitis, and ES is also present. In most dermato-

ses, VCAM-1 and ES show venular endothelial expression. Vascular pericytes in inflamed skin may also stain for VCAM-1; ECs in the same vessel sometimes stain negative. This finding is consistent with our *in vitro* observations of the induction of VCAM-1 staining on VSMCs by TNF- $\alpha$  or IL-1.

VCAM-1 is abundant in the synovitis of rheumatoid arthritis. It is present in venules associated with chronic inflammatory cell infiltrates and also on hyperplastic synovial lining cells. ES is also present; it varies in intensity according to disease activity, and it is localized to ECs. The level of expression of both adhesins is far less in the synovium of osteoarthritis, a condition with fewer inflammatory features (241,243). Psoriatic arthritis, usually indistinguishable from rheumatoid disease with regard to the degree of clinical inflammatory joint findings, also shows less EC ES (244).

VCAM-1 is expressed on venular ECs in cardiac and renal allografts, and its presence correlates with T cell infiltrates (245,246). During rejection of human liver transplants, there is increased expression of ICAM-1 on target structures, such as bile ducts and venous endothelium, as well as on lymphocytes infiltrating the graft (247).

Human coronary arteries and abdominal aortas affected by diffuse intimal thickening and atheromatous plaques show a marked increase in expression of ICAM-1, ES, and, to a lesser extent, HLA-DR/DP on ECs adjacent to subendothelial infiltrates of T lymphocytes and macrophages. This effect contrasts with lower or absent expression of these markers at sites without prominent inflammatory cell infiltrates, and it suggests that cytokines produced by these subintimal infiltrates may activate the endothelium in a manner similar to that observed in the microvasculature at sites of immune inflammation (248).

**IN VIVO ACTIONS:** Subcutaneous injections of TNF- $\alpha$  in baboon skin attempt to stimulate the natural release of this cytokine *in vivo*. These experiments show that ES, ICAM-1, and VCAM-1 expression are induced at post-capillary sites, which concurs with results seen in cultured ECs. Expression of ES at such sites is evident 2 hours after injection, and it correlates highly with neutrophilic exudates. ICAM-1 and VCAM-1 are seen 24 to 48 hours after TNF- $\alpha$  exposure, and they correlate with mononuclear infiltration. Such results support the hypothesis that selective adhesion molecule expression contributes to selective leukocyte extravasation (249,250).

The tissue injury that accompanies hypoxemia and reoxygenation has features of the host response in inflammation, suggesting that cytokines such as IL-1 may act as mediators in this setting. Human ECs subjected to hypoxia elaborate IL-1 activity. There is an increase in the level of IL-1 $\alpha$  mRNA, followed by induction of ES and enhanced expression of ICAM-1 during reoxygenation.

Adherence of leukocytes is increased 3- to 5-fold, and it is partly blocked by antibodies to ES and ICAM-1. Suppression of endothelial-derived IL-1, using antibodies to IL-1 $\alpha$ , specific, antisense oligonucleotides, or the IL-1 receptor antagonist, decreases leukocyte adherence to reoxygenated ECs, thus emphasizing the integral role of IL-1 in the adherence phenomenon.

Mice subjected to hypoxia display increased plasma levels of IL-1 $\alpha$ , induction of IL-1 $\alpha$  mRNA in lung, and enhanced expression of ICAM-1 in pulmonary tissue compared with normoxemic control mice. Thus, hypoxia is a stimulus that induces EC synthesis and release of IL-1 $\alpha$ , and it may result in an autocrine enhancement of adhesion molecule expression (251,252).

*Plasmodium falciparum*-infected erythrocytes isolated from a patient with severe complicated malaria bound to TNF- $\alpha$ -treated human vascular ECs via ES, ICAM-1, and VCAM-1. Attachment of infected erythrocytes to blood vessel walls is understood to be the primary step in the vascular occlusion underlying this disease, in which serum TNF- $\alpha$  levels are characteristically high. ES and VCAM-1 are expressed on brain microvascular endothelium of postmortem brain tissue from patients dying of cerebral malaria (253).

A role for IL-1 and ECs in the neuronal mechanisms related to  $\beta$ -amyloid protein deposition in senile plaques in patients with Alzheimer's disease is suspected. The protein precursor of  $\beta$ -amyloid is expressed on ECs in senile plaques. Its mRNA in human endothelial, neuronal, and brain-derived murine ECs increases when these cells are exposed to IL-1 $\beta$  (254).

Pancreatic carcinoma cells are among a group of neoplastic cells that express the ES ligand, sialyl Lewis (a). Their attachment to activated ECs is thus regulated by cytokines such as IL-1 $\beta$  and TNF- $\alpha$ , which induce endothelial ES (255).

Finally, EC activation by cytokines can also be beneficial. Congenital toxoplasmosis involves infection of umbilical cord vessels as a major route of transmission. IL-1 $\beta$  and TNF- $\alpha$ , in cooperation, inhibit EC replication of *T. gondii*, an obligate intracellular parasite. IFN- $\gamma$  has a similar retardative effect (256).

### Chemokines

Chemokines are a group of 8- to 11-kd proteins produced by ECs as well as leukocytes, fibroblasts, and keratinocytes (206,257). Their primary function is chemoattraction, but stimulation of leukocyte microbicidal activity and the respiratory burst become evident at higher concentrations. They are divided into two families on the basis of their leukocyte predilection and structure. The  $\alpha$ -subfamily, exemplified by IL-8 (neutrophil-activating protein-1), has an amino acid intervening between the

first two cysteines of its amino terminus (i.e., C-X-C), whereas the  $\beta$ -subfamily, exemplified by MCP-1, does not (i.e., C-C) (258,259).

Each chemokine shows some selectivity for a leukocyte species both in vitro and in vivo. IL-8 acts on neutrophils, although there is also some evidence for T cell and eosinophil activity (259-261). MCP-1 is a chemoattractant for human monocytes (262). MIP-1 attracts activated T cells, whereas RANTES (regulated on activation, normal T expressed and secreted) acts on unstimulated T cells, monocytes, and eosinophils (263-265). Furthermore, MIP-1 $\alpha$  acts preferentially on CD8+ lymphocytes, whereas MIP-1 $\beta$  attracts CD4+ cells (263). This chemotactic discrimination, plus that offered by the adhesion molecules, provides the means to selectively control extravasation of each leukocyte subset.

**IL-8 AND NEUTROPHIL TRANSMIGRATION:** Endothelia treated with IL-1 or TNF- $\alpha$  bring about neutrophil transmigration. This effect appears to be at least partly due to stimulation of the endothelium's endogenous production of IL-8, which acts as a chemoattractant if added to the basal EC compartment in an in vitro model of the vessel wall (205,206). Antisera to IL-8 markedly inhibit neutrophil transmigration across activated EC monolayers, and washing the basilar compartment of the vessel wall, which depletes IL-8 from the subendothelial matrix, also prevents neutrophil invasion unless IL-8 is readded (205). IL-8 is less effective in a chemokinetic role (i.e., when placed on both sides of the endothelium) (202).

Given that neutrophils must contact the endothelium for transmigration to occur, it is suggested that IL-8 creates not only a chemotactic gradient, but also a haptotactic gradient of IL-8 molecules over the EC surface, along which neutrophils may move (7,204). Consistent with this proposal, IL-8 binding sites exist on ECs of postcapillary and collective venules and small veins, but they are not found on arteries or capillaries (266). Immunohistochemical analysis of IL-1 $\beta$ -stimulated ECs in vivo reveals IL-8 in association with both the EC monolayer and the underlying interstitium (205). IL-8 may reach these sites by diffusion from perivascular tissues or through local production by endothelium. A soluble chemotactic mechanism alone would be an unlikely method for neutrophil transmigration, because soluble IL-8 inhibits neutrophil-endothelial interactions, it leads neutrophils to shed L-selectin (a molecule involved in their primary rolling attachment to ECs), and it would be continually eroded by virtue of flow dilution (7,267).

Desensitization of neutrophils to IL-8 confirms the existence of another factor involved in the control of transendothelial migration. The procedure decreases neutrophil transmigration through cytokine-stimulated ECs totally. Desensitization to another chemotactic agent, N-

formyl-methionyl-leucyl-phenylalanine (FMLP), creates neutrophils that still respond to an IL-8 gradient, suggesting that the desensitization process does not prevent neutrophil migration. They are, however, inhibited from transmigrating across cytokine-stimulated ECs by 74%, through a putative second, IL-8-independent pathway (268). TNF- $\alpha$  and IL-8 have additive effects on transmigration, which further suggests the existence of an IL-8-independent mechanism (202).

Chemotactic desensitization also demonstrates the dichotomy between adhesion and transmigration. Neutrophils desensitized to IL-8 adhere avidly to ECs due to activation of their CD11b/CD18, but they do not migrate (269). Furthermore, lymphocytes will adhere to TNF- $\alpha$ -treated EC monolayers, but they do not migrate through them (204).

**IL-8 AND NEUTROPHIL ADHESION:** Neutrophils that have established adhesive contact on the endothelium display activation of their  $\beta_2$ -integrins, and they lack L-selectin (7). Soluble IL-8 also causes nonadherent neutrophils to shed L-selectin, and as a result of further, as yet uncertain means, it decreases neutrophil-endothelial interactions (267). Intravenous IL-8 administration to nonhuman primates results in granulocytosis and neutrophil margination in lung, liver, and spleen, but no tissue infiltration (270). Thus, depending on whether more IL-8 is bound or free, neutrophils are either stimulated to or are inhibited from adhering.

Because neutrophil contact with cytokine-activated endothelium may lead to EC damage, IL-8 steers the interactions of these two cells through three possible courses: (a) diapedesis and transmigration, (b) expulsion of granule contents and EC damage, or (c) detachment via soluble IL-8 to reenter the circulating pool (240). The levels of soluble, intravascular IL-8 at a site of inflammation are controlled by the availability of free binding sites, blood flow washing away soluble factors, circulating antibodies to IL-8 (271), and red blood cells, which bind IL-8, rendering it incapable of stimulating neutrophils (272).

**MONOKINES:** MCP-1, MIP-1, and RANTES are the mononuclear cell chemoattractant equivalents of IL-8. Their synthesis is induced in ECs by IL-1, TNF- $\alpha$ , LPS, and thrombin (273,274). IFN- $\gamma$  also induces MCP-1 mRNA, but to a lesser extent (274). MCP-1 protein steadily accumulates from ECs exposed to IL-1 $\beta$  over 48 hours. It has chemoattractant properties for monocytes, and it can activate monocyte  $\beta_2$ -integrins (274,275).

Akin to IL-8, MIP-1 $\beta$  is also present on lymph node endothelium in an immobilized form, and thus it is resistant to loss in the flow of the bloodstream. In vitro immobilization of MIP-1 $\beta$  on proteoglycans assists the binding of T cells to VCAM-1 (276). MIP-1 $\beta$  may therefore control not only the chemotaxis of T cells, but also their ad-

hesion to endothelial VCAM-1. This process is similar to IL-8 activation of neutrophil  $\beta_2$ -integrin, although the mechanism of this effect of MIP-1 $\beta$  is unresolved.

**DISEASE ASSOCIATIONS:** The chemokines are associated with both acute and chronic disease processes. IL-8 appears in the circulation in patients with septic shock, endotoxemia, and after IL-1 administration (277). Bronchioloalveolar lavage IL-8 levels are higher in patients with acute respiratory illnesses in whom the adult respiratory distress syndrome subsequently develops (278), than in those in whom it does not develop. Acute asbestos-induced pleurisy is characterized by an influx of neutrophils. Introduction of crocidolite asbestos or TNF- $\alpha$  into the pleural space leads to the appearance of chemotactic activity for neutrophils, which is inhibited by anti-IL-8 and is accompanied by rapid induction of IL-8 mRNA in mesothelial cells (279).

Extracts of synovium from joints afflicted by rheumatoid arthritis possess diverse chemotactic activities to monocytes, T cells, and neutrophils. mRNA for IL-8, MCP, RANTES, and GRO is expressed in synovial fluid cells and synovial macrophages and fibroblasts. The chemotactic activity can be adsorbed by anti-IL-8 and anti-MCP-1 antibodies. MCP-1 levels are significantly higher in synovial fluid from patients with rheumatoid arthritis than those with osteoarthritis, which is consistent with the relative components of inflammation in the two disorders. The concentration of IL-8 and RANTES mRNA in blood is also less than in synovial fluid cells, which is consistent with the central site of inflammatory activity (280-285).

Circulating antibodies to IL-8 have been demonstrated in patients with rheumatoid arthritis; they correlated strongly with C-reactive protein, number of arthritic joints, and disease activity (271). IL-8 immunostaining is also noted on ECs in the minor salivary glands of patients with Sjögren's syndrome (286).

Minimally modified low density lipoprotein (LDL) induces MCP-1 in human endothelial and smooth muscle cells, and a role in atherosclerosis is further suggested by expression of MCP-1 mRNA and protein in atherosclerotic lesions of rabbits, but not from the intima or the media of normal animals. MCP-1 can be extracted and hybridized from lesional foam cells, but not from alveolar macrophages, sublesional VSMCs, or normal arteries (287,288).

### *Interferon- $\gamma$*

IFN- $\gamma$  is a T-cell product that steers ECs toward a phenotype consistent with chronic inflammation; they express class I and II MHC antigens, and they resemble a high endothelial venule (289). Its intracellular signaling in-

volves phospholipase D-dependent triphasic activation of PKC (290).

**EFFECTS ON ADHESION MOLECULES:** The effects of IFN- $\gamma$  on the EC adhesion molecule profile differ from those of IL-1 and TNF- $\alpha$ . IFN- $\gamma$  stabilizes the surface expression of ES, but it does not induce or prolong its period of synthesis (291). It does not increase PS expression, but ICAM-1 is up-regulated (169,292). IFN- $\gamma$  has a minor part in the induction of the L-selectin ligand compared with TNF- $\alpha$  and IL-1 $\beta$  (181), but it decreases EC expression of CD34, another L-selectin ligand (179).

IFN- $\gamma$  has not been found to induce VCAM-1 on cultured ECs (185), but it does lead to a marked up-regulation of endothelial and dermal dendritic cell VCAM-1 after intradermal injection. In comparison to normal skin, in which VCAM-1 is present on perivascular dendritic cells and some follicular keratinocytes only, VCAM-1 is variably up-regulated on dermal endothelial and dendritic cells in allergic contact dermatitis, atopic dermatitis, lichen planus, and psoriasis, all conditions with increased local IFN- $\gamma$  (293).

**EFFECTS ON MHC EXPRESSION:** Class I MHC appears on ECs exposed to IFN- $\gamma$  (169); the ECs then become antigen-presenting cells for lymphocytes (294). IFN- $\gamma$  increases TAP-1 expression, thus permitting assembly and normal surface expression of the class I MHC molecules. Both class I MHC and TAP-1 are synergistically increased by combinations of TNF- $\alpha$  with IFN- $\gamma$  (196).

Class II HLA-DR antigens are uniquely induced on ECs by IFN- $\gamma$ ; they selectively increase the adhesion of CD4<sup>+</sup> lymphocytes to ECs over other leukocyte populations (289,295,296). Serially passaged EC cultures will stimulate highly purified peripheral blood CD4<sup>+</sup> T cells to proliferate if the EC cultures are pretreated with IFN- $\gamma$  to induce de novo expression of MHC class II molecules (297). T-cell production of IFN- $\gamma$  correlated with the intensity of EC expression of MHC antigen in a rat model of insulinitis (298).

**OTHER ENDOTHELIAL EFFECTS:** Human recombinant IFN- $\gamma$  increases HUVEC monolayer permeability to [<sup>125</sup>I]-labeled bovine serum albumin in a time- and dose-dependent manner. IFN- $\gamma$  and TNF- $\alpha$  or IL-1 produce an increase in permeability greater than that seen with each cytokine alone (299). Migration of lymphocytes through endothelial cell monolayers is also augmented by an endothelial-specific effect of IFN- $\gamma$ . This augmentation affects even prebound lymphocytes; therefore affects migration and not just adhesion (300). The mechanism is not resolved, although IFN- $\gamma$  increases MCP-1 production.

IFN- $\gamma$  decreases EC  $\alpha$ v $\beta$ 3-integrin (the vitronectin re-

ceptor), and it induces  $\alpha$ 1 $\beta$ 1 (192,301). It decreases EC mRNA and protein levels of PDGF and GM-CSF, it increases IL-1 mRNA, and it weakly induces IL-6 production (106,154,302).

### *Hematopoietic CSFs*

IL-3 acts directly on hematopoietic and endothelial cells, and it favors their proliferation, as discussed. Pro-inflammatory effects of excess levels of CSFs have been evident in clinical practice and in animal models (303,304), but they have been ascribed to actions on mature leukocytes, such as those favoring their adhesion to endothelium (305,306) and activation (307-310). ECs have been seen as a source of G-CSF, GM-CSF, and M-CSF when activated by other cytokines (e.g., IL-1, TNF- $\alpha$ , oncostatin-M) (311-313). Modified LDLs produce a similar effect, and because M-CSF binds preferentially to type V collagen (a collagen reported in atherosclerosis), this effect has led to suggestions that CSFs thus produced and acting on leukocytes may have a role in atherosclerosis (314,315).

We and others have observed that resting HUVECs express mRNA for the  $\alpha$ - and  $\beta$ -chains of the IL-3 receptor, and that this is a functional mediator of endothelial interactions with leukocytes (90-92). Previous reports had generally concluded that CSFs do not influence these endothelial properties (e.g., procoagulant activity; production of PAF; expression of ES, PA, or PAI-1) (88,89). Positive reports (e.g., G-CSF augmenting ET-1 production (316), and GM-CSF and M-CSF weakly increasing endothelial ICAM-1 expression) (292,317) are controversial in light of the absence of demonstrable receptors for these cytokines on ECs.

IL-3, however, induces ES surface expression on resting HUVECs, as well as those treated with TNF- $\alpha$ . It supports neutrophil and CD4<sup>+</sup> lymphocyte adhesion (90,92). IL-8 production and neutrophil transmigration across TNF- $\alpha$ -activated HUVEC monolayers are also increased by IL-3 (90). Thus, at least one CSF clearly alters endothelial interactions with leukocytes in vitro.

### *Transforming growth factor- $\beta$*

TGF- $\beta$  is a 25-kd dimer that appears to be a vital anti-inflammatory factor. Three isoforms are known in humans, but consideration is given in this discussion only to TGF- $\beta$ 1. At the cellular level, TGF- $\beta$  has pleiotropic effects on morphogenesis, proliferation, and differentiation (318,319). Lin and Lodish (320) categorize its effects as (a) interruption of cell cycle in mid-to-late G1-phase, thus preventing induction of DNA synthesis and progression into S-phase; (b) induction of ECM and decreased synthesis of matrix-degrading proteinases; and (c) modu-

lation of the secretion of other growth factors and their receptors (320).

Endothelium secretes latent TGF- $\beta$  which undergoes activation in heterotypic co-cultures with other cell types such as pericytes or VSMCs (18,72). Its production in addition appears to be under positive autocrine control and is increased by TNF- $\alpha$  and IL-1 in a synergistic manner (321,322).

**ENDOTHELIAL EFFECTS OF TGF- $\beta$ 1:** TGF- $\beta$  is recognized as a fundamental protein given the multifocal inflammatory disease seen in mice with targeted deletion of the TGF- $\beta$ 1 gene. At birth, these animals show no gross developmental abnormalities, but approximately 20 days later, they succumb to a wasting syndrome accompanied by a mixed inflammatory infiltrate, leading to organ failure in heart, stomach, liver, lung, pancreas, salivary gland, and striated muscle. There are increased numbers of neutrophils and monocytes in peripheral blood, and analysis of cytokine mRNAs from spleen, liver, and lung show increased IFN- $\gamma$ , MIP-1 $\alpha$ , TNF- $\alpha$ , and IL-1 $\beta$  levels (323,324).

Although the mechanisms behind this pathology are not entirely certain and may not be directly applicable to humans, experimental data demonstrate that TGF- $\beta$  negatively modulates the interactions between human ECs and leukocytes therefore contribute to the inflammatory homeostasis of the organism. The basal and TNF- $\alpha$ /IL-1-induced adhesiveness of ECs for neutrophils (325), T lymphocytes (326), monocytes (Litwin MS, et al., unpublished observations), and tumor cells (327) are decreased by TGF- $\beta$ . This decrease is accompanied by a reduction in the EC surface expression of ES; there is no change in VCAM-1 or ICAM-1 (328). Neutrophil transmigration across basal and cytokine-stimulated EC monolayers is also inhibited by TGF- $\beta$ , with an accompanying reduction in EC IL-8 secretion (Smith WC, et al., unpublished observations).

Expression of VCAM-1 on VSMCs in their basal state or after TNF- $\alpha$  stimulation is also inhibited by TGF- $\beta$ . Because active TGF- $\beta$  is produced as a result of coculture of VSMCs and ECs, this close cellular association may be responsible for the lack of VCAM-1 expression on in situ normal VSMCs. Interruption of this contact in atheroma and loss of active TGF- $\beta$  may be important pathogenic events (Gamble JR, et al., unpublished observations).

**TGF- $\beta$  RECEPTORS AND SIGNALING:** There are three cell surface TGF- $\beta$  binding proteins, each reported to be expressed on ECs (74,329). Type I and III receptors are thought to capture TGF- $\beta$  and present it to type II receptors, which are functional transmembrane serine/threonine kinases (320,330). In addition, there are a number of other binding proteins that exist as soluble forms or in the ECM.  $\alpha_2$ -Macroglobulin and  $\beta$ -glycan are believed to

deliver TGF- $\beta$  to its signal transduction receptors, whereas decorin neutralizes TGF- $\beta$  (8). The effects of TGF- $\beta$  on ECs, however, may relate more to expression of downstream components of the signaling pathway than to the type of receptor expressed, because these receptors are found even on TGF- $\beta$ -unresponsive ECs. On other cell types, divergent responses are produced despite expression of the same receptors (331,332).

**DISEASE ASSOCIATIONS:** In several experimental animal diseases modeling human illnesses marked by a significant inflammatory component, administration of TGF- $\beta$  leads to amelioration of both disease and tissue infiltration. In experimental allergic encephalomyelitis TGF- $\beta$  appears to protect against disease relapses. Anti-TGF- $\beta$  antibody increases the incidence and severity of relapses, whereas anti-TNF- $\alpha$  antibody decreases them. TGF- $\beta$  treatment does not influence the appearance of sensitized cells in peripheral blood and lymph nodes, but it does prevent accumulation of T cells in brain and spinal cord (333). Similar benefits from TGF- $\beta$  are claimed in myocardial ischemia/reperfusion injury (334,335), as well as acute and chronic streptococcal-induced arthritis. Histopathological examination of the latter shows reduced inflammatory cell infiltration, pannus formation, and joint erosion (336).

Lipoprotein(a) (Lp[a]) is an LDL-like particle that contains apolipoprotein(a), a molecule with homology to plasminogen. Epidemiological studies have shown significant correlation between blood levels of Lp(a) and coronary/cerebral vascular disease. Lp(a) inhibits generation of TGF- $\beta$  in cocultures of ECs with VSMCs by competing with plasminogen for EC surface binding, thus decreasing the EC plasmin-generating activity. This process may lead to down-regulation of TGF- $\beta$  activation, and, because TGF- $\beta$  is an inhibitor of EC proliferation, adhesiveness for leukocytes, VSMC migration, and VCAM-1 expression, Lp(a) may use this mechanism in the generation of atheromatous lesions (337).

ECs from skin affected by psoriasis show specific unresponsiveness to the inhibitory effects of TGF- $\beta$  on baseline, IL-1-, and TNF- $\alpha$ -induced increases in lymphocyte adhesion, compared with cultured normal dermal microvascular ECs (338). If this finding reflects only swamping of the relatively weak negative signal of TGF- $\beta$  by other more powerful proinflammatory influences, it is still a demonstration of the finely balanced forces in inflammation.

#### Interleukin-4

IL-4 is a product of T cells, mast cells, and bone marrow stroma cells. It has a dominant role in the development of undifferentiated T helper (Th) cells into Th1 and Th2 cells, favoring the Th2 phenotype (339). It also inhibits

several monocyte functions in vitro, including the respiratory burst; adhesion to endothelium and IL-1, TNF- $\alpha$ , IL-6, and IL-8 (340-343).

IL-4 assists endothelial induction of VCAM-1 by IL-1, TNF- $\alpha$ , or IFN- $\gamma$ . Expression of basal and cytokine-induced ES and, to some extent, ICAM-1, is decreased, however, and together with TGF- $\beta$  there is additive inhibition of ES (328,344,345). Through these changes, IL-4 increases EC adhesiveness for T cells, eosinophils, and basophils, but not for neutrophils, because the former express very-late antigen (VLA-4); a ligand for VCAM-1, but neutrophils do not (346,347). Furthermore, eosinophils (but not neutrophils) from individuals with atopic dermatitis migrate through IL-4-pretreated EC monolayers (348).

Both IL-4 and TNF- $\alpha$  increase intracellular cyclic AMP in ECs, but only IL-4 uses this pathway to mediate lymphocyte adhesion. Elevation of cAMP in ECs does not induce VCAM-1, the only identified adhesion molecule induced by IL-4, indicating that an increase in cAMP in EC promotes an as yet unidentified adhesion pathway (349).

IL-4 increases resting endothelial MCP-1 production. It does not further increase IL-1 or TNF-induced MCP-1 mRNA, but there is an increase in secreted MCP-1 with these factors in combination; therefore, monocytes that adhere to the vascular wall by IL-4-induced VCAM-1 may be uniquely positioned to respond to EC-produced MCP-1 (350-352). IL-4 decreases IL-8 production by endothelium (Smith WB, et al., unpublished observations), which further suggests its activity favors mononuclear rather than neutrophil transmigration.

IL-4 may alone be insufficient to mediate leukocyte extravasation in vivo. Studies of monocyte morphology after adhesion to IL-4-treated, VCAM-1-bearing endothelium show that although there are more adherent monocytes, they do not stretch over the surface of ECs, which is thought to be a precursor of their transmigration. In contrast, stimulation of ECs with IL-1 $\alpha$  for 24 hours increases surface expression of both ICAM-1 and VCAM-1, enhances binding of monocytes to ECs, and increases the percentage of EC-bound monocytes with a stretched morphology (353).

IL-4 induces IL-6 production by ECs in synergy with IFN- $\gamma$ , IL-1, and TNF- $\alpha$  (354,355).

### *Interleukin-6*

IL-6 is a T-cell cytokine that acts as a B-cell differentiator, a plasmacytoma growth factor, and a stimulator of hepatic acute-phase reactants. It increases endothelial ICAM-1 expression (292). IL-6 is produced by ECs stimulated with a variety of proinflammatory cytokines, such as IL-1, IL-4, TNF, and IFN- $\gamma$  (221,352,356). Exposure of ECs to mouse hepatitis virus leads to their production of IL-6 (357).

### *Endothelial monocyte-activating polypeptide II*

Endothelial monocyte-activating polypeptide II (EMAP-II) is a 22-kd polypeptide purified to homogeneity from the conditioned medium of murine fibrosarcoma cells based on its ability to induce tissue factor activity in ECs. In addition to procoagulant activity, it induces monocyte migration, and it is chemotactic for granulocytes. Injection into foot pads of mice leads to tissue swelling, with neutrophil infiltration (358).

### **SUMMARY**

The major functions of the endothelium (i.e., renewal, angiogenesis, and interactions with blood components) are subject to the influence of many cytokines (Table 7-1) that often have overlapping, generally redundant effects, but nevertheless a wide spectrum of different actions.

### *Redundancy and pleiotropism among cytokines*

Redundancy among these cytokines is well exemplified by the control of surface adhesion molecules by TNF- $\alpha$ , IL-1, IFN- $\gamma$ , IL-3, and IL-4, or of EC proliferation by FGF, VEGF, SF, IL-3, and TGF- $\beta$ . For instance, TNF- $\alpha$ , IL-1, and IL-4 each encourage interaction of ECs with mononuclear cells by increasing endothelial expression of VCAM-1. TNF- $\alpha$  and IL-1 have the additional capacity to induce the display of ES by ECs and, consequently, their attachment of neutrophils. In direct contrast, IL-4 decreases ES expression by the endothelium, and it restricts its interactions to mononuclear cells and eosinophils.

Stimulation of angiogenesis by FGF and TGF- $\beta$  is another example. Both enhance angiogenesis, but, as described, they appear to have opposite effects on EC division and deposition of ECM. They appear to act in a complementary manner as respective initiators and completors of this process. Therefore, what appears to be redundancy is in fact also specificity and complementary activity created through the varying actions of diverse agents.

Pleiotropism among the cytokines acting on endothelium is also evident. TNF- $\alpha$  and TGF- $\beta$  provide a clear example. Amid their many actions on endothelium is, however, a consistent pattern. TNF- $\alpha$  is the proinflammatory agent that encourages coagulation, adhesion, and chemokine production, whereas TGF- $\beta$  prevents these changes or acts to restore the status quo. A clear aid in defining the understanding of pleiotropic agents, such as TGF- $\beta$ , has been the study of animals with cytokine gene deletions. The multiinflammatory disease of TGF- $\beta$ -deficient mice now awaits further work to determine to what



TABLE 7-1 CYTOKINE ACTIONS ON ENDOTHELIUM

EC Actions	TNF- $\alpha$ , IL-1	IFN- $\gamma$	TGF- $\beta$	IL-4	FGF	VEGF	CSF
Mitosis	↓	↔	↓	↑	↑	↑	↑
Migration	↔	↔	↓	↔	↑	↑	↑
Plasminogen activation	↑	ND	↓	ND	↑	ND	ND
Integrin expression	↑	↑	↔	↔	↑	ND	ND
Angiogenesis	↓	↔	↑	ND	↑	↑	↑
Coagulation	↑	↔	↔	ND	↔	ND	ND
Adhesion molecules	↑	↑	↓	↑↓	↔	↔	↑
Permeability to molecules	↑	↑	ND	↔	↔	↑	↔
Permeability to leukocytes	↑	↑	↓	↔	↔	↔	↑
Cytokine production	↑	↑↓	↓	↑	↔	ND	↔
MHC expression	↑	↑	↔	↔	↔	ND	↔

TNF = tumor necrosis factor; IFN = interferon; TGF- $\beta$  = transforming growth factor- $\beta$ ; IL = interleukin; FGF = fibroblast growth factor; VEGF = vascular endothelial growth factor; CSF = colony-stimulating factor; MHC = major histocompatibility complex; ND = not done.

extent the endothelial effects of TGF- $\beta$  contribute to its overall phenotype. Transgenic methods have not yet been applied to endothelial biology due to the lack of endothelial-specific promoters. Study of the control of ES transcription, which is uniquely expressed on endothelium, offers hope in this direction.

#### *Local availability of cytokines acting on endothelium*

The local availability and source of cytokines are key factors in their relative importance to endothelium. Activated monocytes or lymphocytes, which produce many of the cytokines discussed, are generally features of established, chronic inflammation; thus, their cytokine production would not be expected to begin the first endothelial changes. Mast cells, by virtue of their ubiquity, secretory granule storage (i.e., holding TNF- $\alpha$ ), and responsiveness to neural stimuli, are suspected of being the key initiating cell. In delayed-type hypersensitivity reactions in human skin, degranulation of mast cells situated about superficial vessels is the first ultrastructural change seen; it precedes inflammatory cell accumulation by 16 hours (151).

Cytokine availability is influenced by the ECM, which may provide binding sites that function as reservoirs or aids to receptor interaction (8). The presence of binding sites is clearly important for the chemokines; as bound surface molecules, they favor leukocyte haptotaxis, but as free molecules, they inhibit leukocyte adhesion to ECs. ECM-EC interactions can also trigger the same intracellular signals evoked by cytokines, and the ECM is another

area in which endothelial cell biologists will find fertile ground.

#### *Signaling*

Although cytokines are important signals for ECs, other means of communication are increasingly being understood. Molecules assigned one particular function have been discovered to have a second signaling function. For example, CD31 (PECAM-1) was viewed only as an intercellular junction molecule with a role in leukocyte transmigration, but its ligation selectively assists interaction between the  $\alpha 4 \beta 1$ -integrin found on leukocytes and VCAM-1 (359). Mechanical displacements from the bloodstream lead to changes in cell biochemistry. Flow or shear stress is transduced by ECs into the induction of c-fos, PDGF, and activated factor X expression. Circumferential tensile stress due to blood pressure leads to thickening of the vascular wall (360-362).

The signaling pathways of cytokines, as well as the signals that follow ligation of surface molecules or perceptible displacement of cell membranes, are likely to assume increasing importance. The cyclosporine revolution in clinical medicine has clearly shown that the pathways for intracellular communication are central to an understanding of cytokine actions, and that they are likely to be promising sites for clinically oriented interventions. TNF receptor-binding mutants with decreased endothelial proinflammatory actions that retain antitumor properties offer the promise of isolating the actions of other pleiotropic cytokines and potentially applying these findings selectively to clinical practice (219).

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COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

IN THE MATTER OF: Australian  
Patent Application 696764  
(73941/94). In the name of:  
Human Genome Sciences Inc.

- and -

IN THE MATTER OF: Opposition  
thereto by Ludwig Institute for Cancer  
Research, under Section 59 of the  
Patents Act.

Annexure GBC-11

This is **Annexure GBC-11** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

Gary Baxter Cox  
**Gary Baxter Cox**

WITNESS:

PeYTEE K. HONG  
Patent Attorney

PEYTEE K. HONG

# Regulation of In Vitro Capillary Tube Formation by Anti-Integrin Antibodies

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**Abstract.** Human endothelial cells are induced to form an anastomosing network of capillary tubes on a gel of collagen I in the presence of PMA. We show here that the addition of mAbs, AK7, or RMAC11 directed to the  $\alpha$  chain of the major collagen receptor on endothelial cells, the integrin  $\alpha_2\beta_1$ , enhance the number, length, and width of capillary tubes formed by endothelial cells derived from umbilical vein or neonatal foreskins. The anti- $\alpha_2\beta_1$  antibodies maintained the endothelial cells in a rounded morphology and inhibited both their attachment to and proliferation on collagen but not on fibronectin, laminin, or gelatin matrices. Furthermore, RMAC11 promoted tube formation in collagen gels of increased density which in the absence of RMAC11 did not allow tube formation. Neither RMAC11 or AK7 enhanced capillary formation in the absence of PMA. Lumen structure and size were also altered by antibody RMAC11. In the absence of antibody the majority of lumina were formed intra-

cellularly from single cells, but in the presence of RMAC11, multiple cells were involved and the lumen size was correspondingly increased. Endothelial cells were also induced to undergo capillary formation in fibrin gels after PMA stimulation. The addition of anti- $\alpha_2\beta_1$  antibodies promoted tube formation in fibrin gels and inhibited EC adhesion to and proliferation on a fibrinogen matrix. The enhancement of capillary formation by the anti-integrin antibodies was matrix specific; that is, anti- $\alpha_2\beta_1$  antibodies only enhanced tube formation on fibrin gels and not on collagen gels while anti- $\alpha_1\beta_1$  antibodies only enhanced tubes on collagen and not on fibrin gels. Thus we postulate that changes in the adhesive nature of endothelial cells for their extracellular matrix can profoundly effect their function. Anti-integrin antibodies which inhibit cell-matrix interactions convert endothelial cells from a proliferative phenotype towards differentiation which results in enhanced capillary tube formation.

**N**EW blood vessel formation is an essential event in embryogenesis and wound healing. However, little is known about the steps which induce flat, static endothelial cells (EC) to undergo differentiation leading to a new capillary bed. Recently, a number of in vitro assays have been established which are thought to mimic angiogenesis and these have provided insight into possible mechanisms. EC initially undergo a spatial reorientation towards the angiogenic stimulus, invade and disrupt the extracellular matrix (ECM) by production of matrix degrading enzymes such

as plasminogen activator and collagenase and undergo tube formation and extension via EC proliferation (for review see Folkman and Handenschild, 1980; Folkman, 1986). Thus, measurement of EC realignment, enzyme secretion, and cell proliferation are taken as indicators of EC differentiation and tube formation. Using such assays, it is clear that a number of factors including basic fibroblast growth factor, TGF- $\beta$ , and tumor necrosis factor (Folkman and Klagsbrun, 1987) (termed angiogenic factors), can stimulate angiogenesis. In addition to the requirement for an angiogenic factor, in vitro capillary formation is also dependent on the correct matrix. Endothelial cells, plated on a two-dimensional matrix of ECM proteins or on plastic, form capillary tubes slowly (2–3 wk) (Kubota et al., 1988; Iruela-Arispe et al., 1991). However, when plated onto a gel of the ECM proteins, tubes form within 24 h (Kubota et al., 1988). Isolated components of the ECM, such as collagen or fibrinogen, when gelled are also able to induce the formation of capillary tubes (Monte-

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**1. Abbreviations used in this paper:** EC, endothelial cells; ECGS, endothelial cell growth supplement; ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cell; MVEC, microvessel EC; uPA, urokinase plasminogen activator.

sano et al., 1985). In collagen and fibrin gels the process of capillary formation is enhanced by the activation of the EC with the tumor promoter, PMA (Montesano and Orci, 1985; Montesano et al., 1987). Thus, even in the presence of an angiogenic factor, and a permissive milieu, an additional signal is required and this can be provided by PMA.

Cell surface molecules mediating adhesion to either neighboring cells or to substrates are likely to play a key role in angiogenesis. The integrins are a family of cell surface molecules which mediate the attachment of cells to the ECM and to other cells. At least 19 different cell-surface  $\alpha\beta$  heterodimers have been identified, some of which mediate adhesion to ECM proteins such as laminin, collagen, fibrinogen, and fibronectin (Ruoslahti and Pierschbacher, 1987; Hynes, 1987; Albelda and Buck, 1990). EC express five of the six  $\beta$  integrins ( $\alpha_1\beta_1$ ,  $\alpha_3\beta_1$ ,  $\alpha_5\beta_1$ ,  $\alpha_v\beta_1$ , and  $\alpha_6\beta_1$ ) although there is some heterogeneity in the level of expression depending on the source of the EC (DeFilippi et al., 1991a). The major collagen receptor on EC is  $\alpha_3\beta_1$  (Albelda et al., 1989; Languino et al., 1989) which can also mediate binding to laminin (Kramer et al., 1990). EC also express  $\alpha_v\beta_3$  which mediates adhesion to fibrinogen (Albelda et al., 1989; Cheresh and Spiro, 1987) as well as laminin (Kramer et al., 1990) and vitronectin (Cheresh and Spiro, 1987).

We describe here for the first time that anti-integrin antibodies directed to the receptors mediating attachment to the tube-permissive matrices of collagen I and fibrin enhance capillary formation as measured by tube number, length, and thickness. Anti-integrin antibodies enhance tube thickness by increasing the number of cells involved in lumen formation. These results suggest that capillary tube formation is dependent on the interaction of EC with the ECM and that restriction of specific cell-matrix interactions can enhance the extent of capillary formation.

## Materials and Methods

### Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase treatment as described (Wall et al., 1978). The cells were cultured in 25 cm<sup>2</sup> gelatin (Eastman Kodak Co., Rochester, NY)-coated flasks (Costar Corp., Cambridge, MA) in M199 with Earle's Salts, 20 mM Hepes, 20% FCS (Cytosystems, Sydney), sodium bicarbonate, 2 mM glutamine, nonessential amino acids, sodium pyruvate, fungizone, penicillin, and gentamycin (HUVEC medium). Cells were grown at 37°C, 5% CO<sub>2</sub>. Within 2–4 d the HUVEC formed a confluent monolayer and were then harvested by trypsin-EDTA treatment and transferred to a 75-cm<sup>2</sup> gelatin-coated flask. 50 µg/ml of endothelial cell growth supplement (ECGS, Collaborative Research, Bedford, MA) and 50 µg/ml of heparin (Sigma Chem. Co., St. Louis, MO) were added. Cells were passaged (1:2 split) every 3–4 d, and were used between passage 2 and 6. Microvessel EC (MVEC) were prepared from neonatal foreskins according to the method of Marks et al. (1985). Cells were frozen in liquid nitrogen at  $1-2 \times 10^6$ /vial at passage 2–6 and thawed as required. Cells were grown to confluence before use. Medium for growth and maintenance of these EC was M199 with Earle's Salts, 25 mM Hepes, 50% human serum, sodium bicarbonate, 2 mM glutamine, fungizone, penicillin, streptomycin,  $3.3 \times 10^{-4}$  M cAMP, ECGS (50 µg/ml), and heparin (50 µg/ml).

### Collagen Gel Capillary Assay

Bovine Type I collagen (Celtrix Laboratories, Palo Alto, CA) gel was prepared by simultaneously raising the pH and the ionic strength of a collagen solution, using a modification (Greenburg and Hay, 1982) of the original

method described by Elsdale and Bard (1972). Seven volumes of ice cold collagen solution (3 mg/ml) was mixed with 1 vol of 10× concentrated PBS, pH 7.4, and 2 vol of sodium bicarbonate (11.76 mg/ml) on ice. One hundred µl of the mixture was aliquoted into 96 well flat-bottomed microtiter trays (Nunc, Roskilde, Denmark) and allowed to gel for 10–20 min at 37°C. For more rigid collagen gels, the mixture was allowed to gel for at least 1 h at 37°C. After gel formation, EC, which were removed from confluent monolayers by trypsin treatment, were plated down onto the gel at a concentration of  $6.4 \times 10^4$  cells/160 µl in HUVEC or MVEC medium with ECGS and heparin. As indicated 20 ng/ml PMA (Sigma Chem. Co.) was added to some wells. In some assays, as indicated, cells together with PMA were resuspended in the collagen before gelling (modification of method by Madri et al., 1988).

### Fibrin Gel Capillary Assay

Three dimensional fibrin gels were prepared as previously described by Montesano et al. (1985). One hundred µl of plasminogen free fibrinogen, 3 mg/ml in PBS (Sigma Chem. Co.) was placed into 96 well flat-bottomed microtiter wells (Nunc) and clotted by the addition of 2 µl of 1 U/ml thrombin (Parke Davis Pty Ltd., Adelaide, Australia) in PBS. The mixture was allowed to gel for ~2 min at 37°C before addition of EC. Cell numbers and conditions were the same as that described for the collagen gel assay.

### Quantification of Capillary Formation

Tube formation was assessed at several different focal plains through the gel. The extent of capillary tube formation was judged in relation to the amount of EC monolayer and to the number, width, and length of the tubes formed. Based on these criteria values from + to ++++ were assigned. Tube formation was also quantified from high power photographs. At least two photographs from random fields from each microtiter well (duplicate wells were set up for each group) were taken. Areas of the well were avoided where the meniscus gave a distortion of the optics. We defined tubes as straight cellular extensions joining two cell masses or branch points. The minimum width of tubes was measured and is given as the width. From the photographs, counts were made of the number, length, and width of tubes.

### Proliferation Assay

The mitogenic response of EC to different stimuli was measured using a spectrophotometric assay (Oliver et al., 1989). Cells ( $5 \times 10^3$ ) were plated onto matrix-coated flat-bottomed microtiter trays (Nunc), 150 µl/well in HUVEC medium either with or without ECGS, and heparin. After 3 d, medium was removed and the cells fixed in 10% formal saline for 30 min. One hundred µl of methylene blue (1% wt/vol, in 0.01 M borate buffer, pH 8.5) was then added to each well, incubated for 30 min, the stain flicked off, and the cells washed 3–4 times in borate buffer. The dye was released by addition of ethanol, 0.1 M HCl solution (1:1) with a brief shaking. The optical density at 630 nm (OD630 nm) was then determined. The percentage proliferation was calculated based on the OD630 of the no antibody control group normalized to 100%. Experiments showed that there was a linear relationship between the OD630 nm and the increase in cell number and that as low as  $10^3$  cells/well could be detected. Similar results were obtained using the methylene blue assay and the uptake of [<sup>3</sup>H]thymidine (data not shown).

### Cell Attachment Assays

Microtiter plates were coated with either collagen I (50 µg/ml), gelatin (1%), or fibronectin (100 µg/ml) for 30 min at room temperature. Fibrin-coated microtiter wells were formed by thrombin treatment (1 U/ml) of fibrinogen which had been previously added to wells. Wells were washed twice with PBS and  $5 \times 10^3$  cells were added per well in 50 µl of HUVEC medium without FCS. Plates were incubated at 37°C for the indicated times and cell attachment quantitated using the methylene blue assay (as outline above).

### Collagenase Assay

Measurement of active collagenase was performed according to the method of Nethery et al. (1986). Microtiter wells were coated with 0.7 mg/ml collagen I solution (Collaborative Research Inc.), rinsed in water, and air dried. Cell supernatants were treated with 0.5 mg/ml trypsin at 37°C followed by treatment with soybean trypsin inhibitor (Sigma Chem. Co.) at 5 mg/ml.

Collagenase samples (CLS-1 Worthington Biochem. Corp., Freehold, NJ) were prepared at concentrations from 1 to 1,000  $\mu\text{g/ml}$  in assay buffer (50 mM tris-HCl, 100 mM NaCl 10 mM  $\text{CaCl}_2$ , pH 7.5) and were used to generate a standard curve. One hundred  $\mu\text{l}$  of samples and standards were then added to the collagen-coated microtiter wells and incubated for 16 h at 37°C. The wells were washed and stained using 0.25% Coomassie blue R-250 (Biorad Labs., NSW, Australia) for 25 min at room temperature. The stain was removed, wells washed in water, dried, and the absorbance at 590 nm determined. The level of collagenase present in the sample was inversely proportional to the optical density reading at 590 nm. The level of detection was 10 ng/ml.

### Monoclonal Antibodies

RMAC11 binds the  $\alpha$  chain of the  $\alpha_2\beta_1$  integrin complex on endothelial cells and fibroblasts and QE2E5 binds the  $\beta_1$  chain (O'Connell et al., 1991). Preclearing and immunoprecipitation experiments show that AK7 also recognizes the  $\alpha$  chain of the  $\alpha_2\beta_1$  complex (Mazurov et al., 1991). RMAC11 (IgG2a), QE2E5 (IgG2b), and AK7 (IgG1) were purified from ascites fluid using a mAb Trap G Sepharose column (Pharmacia LKB, NSW, Australia). Fab<sub>2</sub> fragments of RMAC11 were prepared by pepsin digestion and Fab fragments by papain digestion of the IgG according to the method of Harlow and Lane (1988). P4C10, an IgG<sub>1</sub> antibody directed to the  $\beta_1$  integrin chain (Carter et al., 1990) was kindly provided by Dr. W. G. Carter, Fred Hutchinson Cancer Research Center, Seattle, WA. Another antibody, 61.2C4, has been shown to be directed to the  $\beta_1$  chain (Gamble, J. R., and M. A. Vadas, unpublished observations). LM609 and 13C2, both anti- $\alpha_2\beta_1$  antibodies, were kindly provided by Dr. David Cheresch, Scripps La Jolla, CA and Dr. Michael Horton, Department of Haematology, St. Bartholomew's Hospital, London, respectively.

### Analysis by Flow Cytometry

EC were plated on collagen gels either in the presence or absence of PMA. 24 h later, cells either as tubes or as a monolayer were extracted by treatment of the gels for 30 min in 2 mg/ml collagenase at 37°C. Cells were then trypsin-treated for 5 min to obtain single cell suspensions. Cells were stained with the appropriate antibodies for 30 min at 37°C followed by a rabbit anti-mouse-FITC antibody for 30 min at 4°C. Cells were washed three times in PBS and diluted in fixative (2% glucose, 5 mM sodium azide, 1% formaldehyde) before analysis on an Epics Profile Analyzer. Ten thousand cells were analyzed.

### Microscopy

At 24 h after stimulation with PMA or PMA and RMAC11, culture medium was removed from the culture wells and replaced with 2% paraformaldehyde, 2.5% glutaraldehyde in sterile PBS (pH 7.4). Cultures were fixed in this solution for 12 h at room temperature (20°C). After 12 h, fixative was washed out with PBS, at least 10 changes every 10 min, and the cultures postfixed for an additional 12 h in 1% osmium tetroxide in PBS. The osmium tetroxide was washed from the cultures, at least 10 changes every 10 min. Cultures were then dehydrated for three 20-min periods in each of a graded series of alcohol (70, 80, 95, and 100% ethanol in twice distilled water), and then for 60 min in each of two 100% ethanol washes. Cultures were transferred to vials containing 100% acetone. After three changes of acetone, each over 1 h, cultures were infiltrated overnight with a 1:1 mixture of acetone and epon-araldite. The next day the acetone was allowed to evaporate off in a fume hood, cultures were placed in fresh epon-araldite for ~6 h, and finally transferred to a mould in a 70°C oven to polymerize overnight. All procedures were carried out at room temperature.

For light microscopic examination, sections were cut 1  $\mu\text{m}$  thick with a dry glass knife using an LKB ultratome. The sections were mounted on glass slides and stained with 1% toluidine blue in 1% borax. Sections were examined with a Leitz Orthoplan microscope. When endothelial cells containing lumens were seen the block was trimmed for thin sectioning (EM). For EM, thin sections of silver interference color were cut with a Swiss diamond knife (Diatome Ltd., Fort Washington, PA), and picked up on clean uncoated 200-mesh copper grids. Sections were stained for 5 min with 2% uranyl acetate in 50% ethanol, and for 5 min with lead citrate. Micrographs were taken on a Philips 410 electron microscope at 80 Kv. Magnifications were determined by means of a carbon grating replica. At both the light microscope level and the electron microscope level, serial sections up to 30 each (1  $\mu\text{m}$  thick or silver interference color) were taken to establish that the lumens were continuous through the endothelial cell.

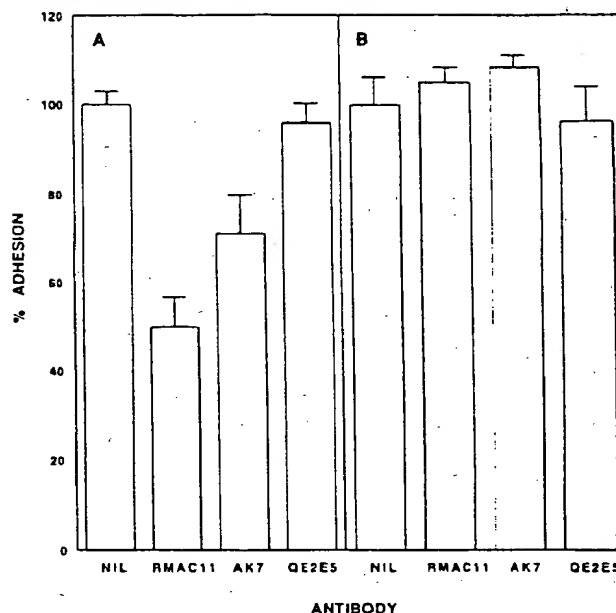


Figure 1. Anti- $\alpha_2\beta_1$  antibodies partially inhibit EC adhesion to collagen I and not to fibronectin.  $5 \times 10^3$  EC in 50  $\mu\text{l}$  of serum-free medium were added to microtiter wells which had previously been coated with either collagen I (A) or fibronectin (B). The antibodies, as indicated, were added at a final concentration of 30  $\mu\text{g/ml}$ . The plates were incubated at 37°C for 2 h, washed, and the number of attached cells assessed by the methylene blue assay (as outlined under Proliferation Assay in Materials and Methods). The results were normalized where 100% adhesion is taken as the OD<sub>630 nm</sub> in wells without antibody. The results show the mean of triplicate wells for each group in one experiment representative of five similar experiments.  $p < 0.001$  for groups with RMAC11 and AK7 compared to no antibody group on the collagen I matrix.

### Statistics

Significance was determined by the ANOVA test for analysis of variance or by the unpaired *t* test.

### Results

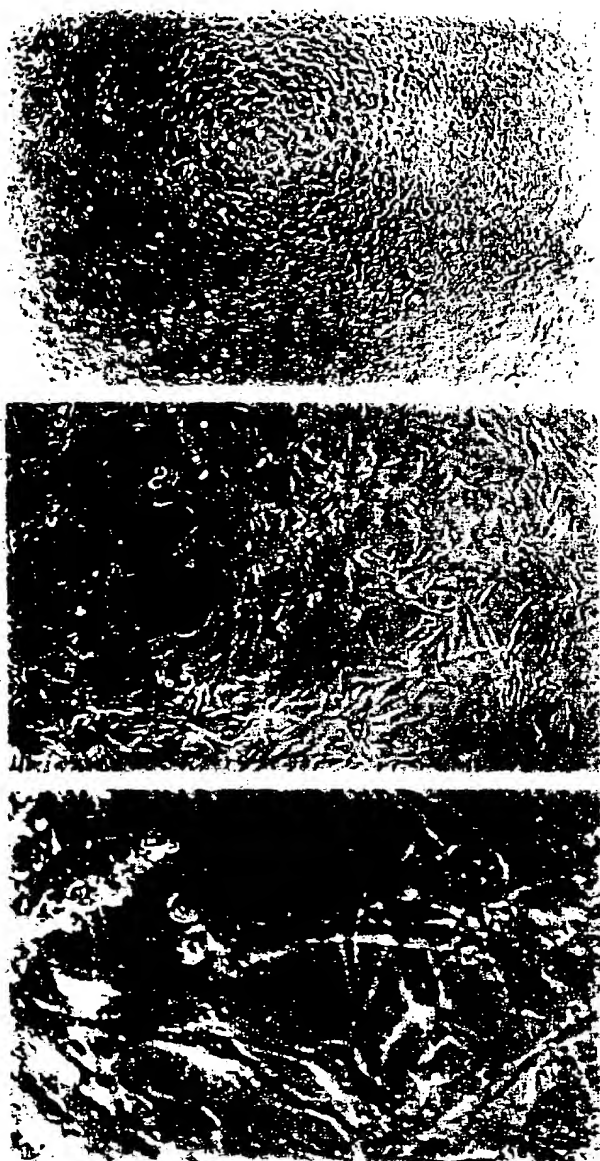
#### Inhibition of Endothelial Cell Adhesion by Anti-Integrin Antibodies

Adhesion of HUVEC to a collagen I matrix was inhibited, although only partially, by RMAC11 and AK7 (Fig. 1 A) but the antibodies had no effect on HUVEC attachment to fibronectin (Fig. 1 B) or gelatin (data not shown). Cells did not attach to laminin in the absence of FCS. The antibody QE2E5 which binds to the  $\beta_1$  chain had no effect on the attachment of cells to any of the matrices tested. However, two other  $\beta_1$  specific antibodies, P4C10 and 612C4, almost totally inhibited HUVEC adhesion to collagen (and fibronectin) (data not shown).

#### HUVEC Plated onto Collagen Gels Are Induced to Form Capillary Tubes

EC, when plated onto a gel of collagen are induced to form a capillary network within the collagen gel in the presence of PMA (Fig. 2, B and C) (Montesano et al., 1983; Mon-





**Figure 2.** EC plated on collagen I gel in the presence of PMA are induced to form capillary tubes.  $6.4 \times 10^4$  EC/well in 160  $\mu$ l of HUVEC medium either in the absence (A) or presence (B) of 20 ng/ml PMA were plated onto a gel of collagen I formed in microtiter wells. The results were visualized 24 h after cell plating. C is a high power photograph of group B. Photographs show representative fields of one well of duplicate wells set up for each group: Magnification (A)  $\times 60$ , (B)  $\times 60$ , and (C)  $\times 240$ .

tesano and Orci, 1985). Realignment of the EC, cell invasion into the gel, and the beginning of cell elongation are evident  $\sim 8$  h after plating of the cells onto the gel, and tube formation was clearly visible by 12 h. Very little, if any, tube formation occurs in collagen gels in the absence of PMA or with addition of dimethylsulphonic acid, used as a carrier for PMA; the cells are maintained as a monolayer on top of the gel and little invasion into the gel is seen (Fig. 2 A). Tube formation was highly dependent on the concentration of cells

plated onto the collagen gel, no tubes being observed with less than  $1-2 \times 10^4$  cells/well suggesting that cell-cell contact is important.

Analysis by EM of EC, 24 h after plating on collagen gels with the addition of PMA showed the presence of vacuole-like structures similar to those described by Folkman and Haudenschild (1980) (Fig. 3 A). A continuous membrane surrounded each vacuole which was either empty or filled with amorphous material. Fusion of these vacuoles with the plasma membrane was occasionally observed. Serial sectioning to  $>30 \mu$ m and visualization at the light microscopy level showed a continuous vacuole-like structure confirming that these were indeed lumina. At least 70% of all cells visualized showed vacuolization. Only the occasional lumen was formed from multiple cells.

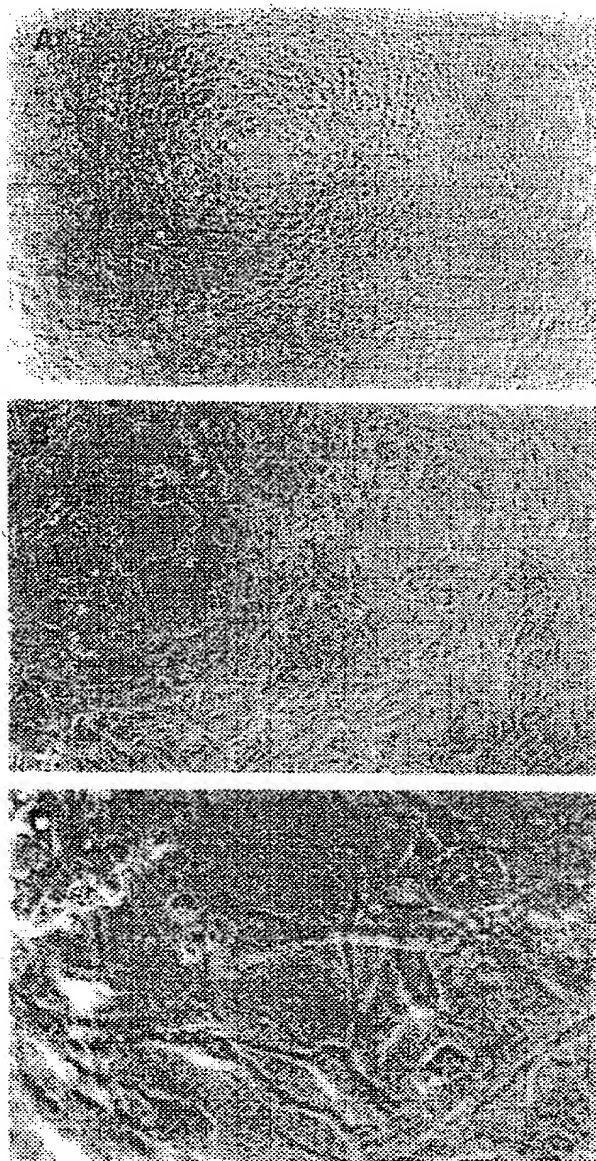
#### *Effect of Anti-Collagen Receptor Antibodies on Capillary Formation*

EC were plated onto collagen gels in the presence of PMA with increasing concentrations of antibodies RMAC11, AK7, and QE2.E5. Capillary formation was assessed 24 h later. Fig. 4 A shows the capillary tube formation taking place with PMA alone. No change in the extent of tube formation was seen with the addition of QE2.E5 (Fig. 4 B). However, a more extensive capillary network was seen in wells containing RMAC11. The tubes appeared longer and wider, and less monolayer was evident (Fig. 4 C). Similar results were obtained with AK7, P4C10, and 612C4 and with EC derived from neonatal foreskins (data not shown). The enhancement of tube formation with RMAC11 over PMA alone was clearly evident in the high power photographs (Fig. 5, A-F). Tube number, length, and width were quantified from high power photographs and results from four separate experiments were analyzed and pooled and are shown in Fig. 6. The anti- $\alpha_2\beta_1$  antibody RMAC11 clearly enhanced the length (A), width (B), and number (C) of tubes.

EM showed that in the presence of RMAC11, cells were seen in larger aggregates suggesting cell-cell adhesion was promoted (not shown). Furthermore, larger lumina were evident compared to PMA group only, the majority of which appeared to be formed from multiple cells as shown in Fig. 3 B. Inter cellular junctions are clearly visible at the EM level and by analysis of these junctions the lumen shown in Fig. 3 B is formed from five EC. Analysis of thin sections (silver interface color) taken through gels showed that with PMA  $7.3 \pm 5.7\%$  (mean  $\pm$  SEM) of lumina were formed from single cells (i.e., intracellular lumen, Fig. 3 A). The remaining lumina were formed from two or three cells. In contrast, in the presence of RMAC11,  $8.3 \pm 7.4\%$  of lumina were formed from three or more cells (values were obtained from four separate experiments with 50 lumen examined in each group in each experiment). Thus the increase in capillary thickness appears to be due to the conversion from intracellular to multicellular lumina.

Maximum levels of tube formation were seen with 30  $\mu$ g/ml RMAC11 (Table I) but some enhancement over the level seen with PMA alone was normally observed with 3  $\mu$ g/ml RMAC11. Tube length, width, and number were assessed visually through different focal plains of the gel and assigned values from + to + + + +. Tube length, width, and number were also measured from high power photographs.





**Figure 2.** EC plated on collagen I gel in the presence of PMA are induced to form capillary tubes.  $6.4 \times 10^4$  EC/well in 160  $\mu$ l of HUVEC medium either in the absence (A) or presence (B) of 20 ng/ml PMA were plated onto a gel of collagen I formed in microtiter wells. The results were visualized 24 h after cell plating. C is a high power photograph of group B. Photographs show representative fields of one wall of duplicate wells set up for each group. Magnification (A)  $\times 60$ , (B)  $\times 60$ , and (C)  $\times 240$ .

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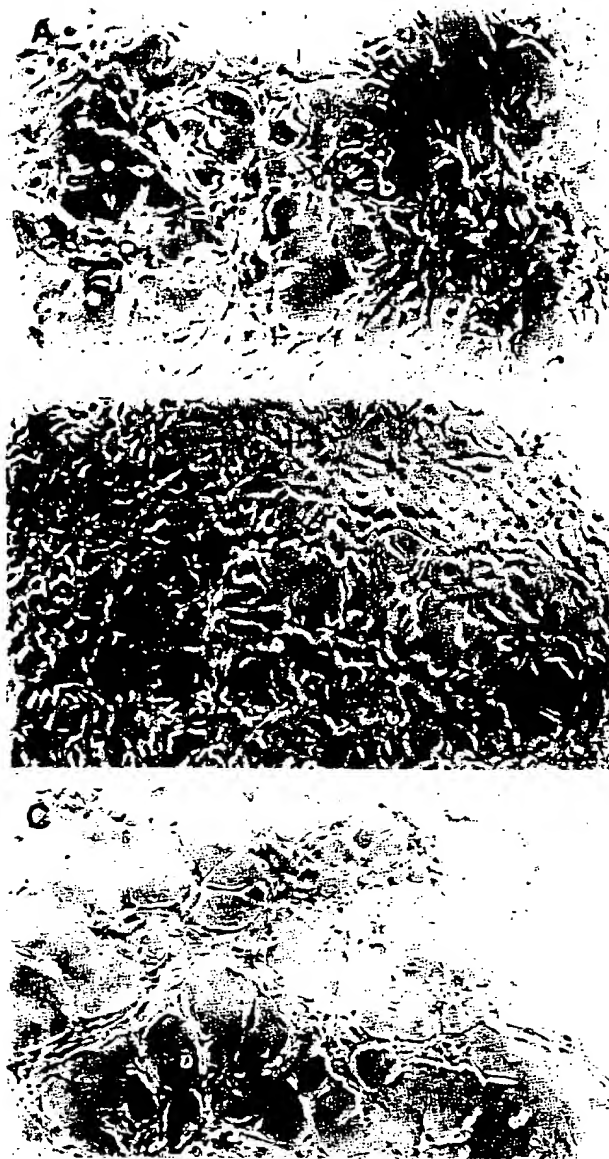
Maximum levels of tube formation were seen with 30  $\mu$ g/ml RMAC11 (Table I) but some enhancement over the level seen with PMA alone was normally observed with 3  $\mu$ g/ml RMAC11. Tube length, width, and number were assessed visually through different focal plains of the gel and assigned values from + to + + + +. Tube length, width, and number were also measured from high power photographs.



**Figure 3.** Formation of lumen-containing capillaries in collagen gels. EC in the presence of 20 ng/ml PMA (A) or PMA and 30  $\mu$ g/ml RMAC11 (B) were plated onto a collagen gel. 24 h later the cells were fixed, embedded in epon-araldite, and sectioned for EM. Magnification (A)  $\times 7490$  and (B)  $\times 4000$ . C, collagen; L, lumen. Numbers 1-5 relate to cell junctions.

As is seen in Table I, the two assessments for capillary formation showed a good correlation. Analysis of EC by flow cytometry showed that 30  $\mu$ g/ml of RMAC11 fully saturated the  $\alpha_2\beta_1$  receptor binding sites (data not shown). With

AK7, an enhancement of tube formation was also seen over the concentration range of 3-30  $\mu$ g/ml. However, the extent of enhancement was never as great as that seen with RMAC11.



**Figure 4.** Anti- $\alpha_2\beta_1$  antibodies enhance capillary tube formation in collagen gels. EC were plated onto collagen gel in the presence of 20 ng/ml PMA. To group B, QE2E5 was added at plating and to group C, RMAC11 both at a final concentration of 30  $\mu$ g/ml. Groups were assessed for tube formation 24 h later. Each photograph shows a representative field taken from one well of duplicate wells set up for each group. Similar results have been obtained more than 20 times using different EC lines. Magnification  $\times 60$ .

The effect observed by RMAC11 and AK7 is unlikely to be due to nonspecific effects via Fc receptor mediated events since Fab<sub>2</sub> fragments of RMAC11 exhibit a similar enhancement of tube formation as the whole Ig (data not shown). Furthermore, since Fab' fragments of RMAC11 were also able to enhance tube formation (data not shown), cross-linking of the antigen is also not likely to be responsible for the enhancement.

Pretreatment of the HUVEC with RMAC11 or AK7 for 15

min either at room temperature or 37°C followed by washing to remove unbound antibody, did not result in enhanced tube formation (data not shown).

The enhancement of tube formation was dependent on the addition of RMAC11 or AK7 within the first 2 h of cell plating. If the antibodies were added after this, no enhancement of tube formation was evident (data not shown).

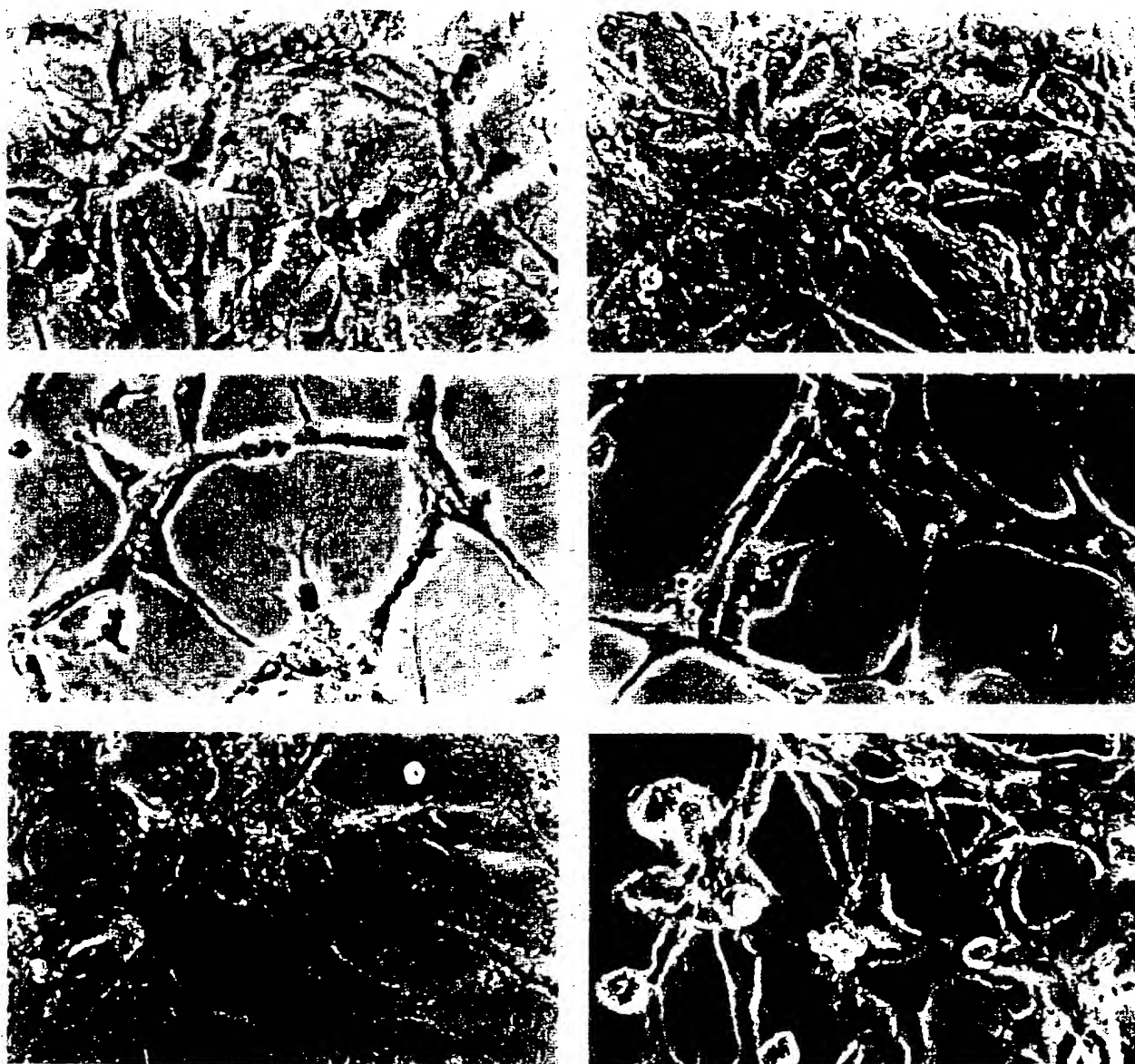
Angiogenesis can also be induced in collagen gels in the presence of PMA by resuspending the cells within the gels rather than layering them on top of the gel (Madri et al., 1988). Pretreatment of EC with PMA and RMAC11 before resuspension within the gel led to enhancement of tube formation over that seen with PMA alone (data not shown). Thus, the anti- $\alpha_2\beta_1$  antibodies were able to enhance tube formation whether the EC contact collagen in a polarized fashion or whether the cells are totally surrounded by the collagen matrix.

#### **Anti- $\alpha_2\beta_1$ Antibodies Induce Capillary Tube Formation in Rigid Gels**

The ability of RMAC11 and AK7 to enhance capillary tube formation was most strikingly evident when EC were plated onto rigid collagen gels formed after the collagen was gelled for 1 h at 37°C. Even in the presence of PMA, very little, if any, tube formation is seen with these rigid gels and the cells maintain a flat cobblestone appearance on top of the gel. However, the addition of RMAC11 (or AK7) with PMA overcomes the inhibitory effect caused by the rigidity of the gel; the cells invade into the gel and capillary tubes and an anastomosing network are visible (Fig. 7, A-D) although not to the same extent as is normally seen on less rigid gels. Measurements of tube numbers from random high power fields from five separate experiments on rigid gels each using a different HUVEC line were made. The mean tube number with PMA alone was  $1.0 \pm 0.4$  (mean  $\pm$  SEM) and with RMAC11 was  $39.2 \pm 4.08$  ( $p < 0.0005$ ) clearly demonstrating the promotional activity of RMAC11. One possibility for the antibodies enhancing the ability of the EC to breakdown and invade the gel is by an increase in the synthesis of matrix-degrading enzymes such as collagenase. However, no change in the total level of active collagenase was observed either in normal gels or in rigid gels. The level of detection of collagenase by the assay used (see Materials and Methods) was 10 ng/ml. In a representative experiment, the level of collagenase induced on collagen gels in the presence of 20 ng/ml PMA was  $112 \pm 5.9$  and with PMA and RMAC11  $120 \pm 2.0$  (mean  $\pm$  SEM,  $n = 3$ ).

#### **Effect of Anti- $\alpha_2\beta_1$ Antibodies on EC Morphology**

As was shown in Fig. 1, the addition of RMAC11 and AK7 to HUVEC cultured on 2-D substrates of collagen, but not on gelatin or fibronectin, resulted in a decrease in the number of cells attached when measured 2 h after plating. No differences were seen in the number of cells attached when measured at 4, 6, or 24 h after plating. On collagen gels, in the presence of QE2E5, the cells had become flattened and were beginning to adopt their characteristic cobblestone morphology (Fig. 8 A) when viewed 2 h after plating. In the presence of RMAC11 (or AK7), cells remained rounded (Fig. 8 B) for  $\sim 2$ -3 h after which time they flattened and no differences from control wells were seen.



**Figure 5.** High power photographs of capillary tubes formed on collagen gels in the presence of anti-integrin antibodies. EC were plated onto collagen gel in microtiter wells with 20 ng/ml PMA. *A* and *B* show one EC line, *C* and *D* another line, and *E* and *F* a third line. To one group (*A*, *C*, and *E*), 30  $\mu$ g/ml of QE2E5 was added and to the other group (*B*, *D*, and *F*), 30  $\mu$ g/ml RMAC11. All wells were incubated for 24 h. Each photograph shows a representative field taken from one well of duplicate wells set up for each group. Magnification  $\times 264$ .

#### **Anti- $\alpha_1\beta_1$ Antibodies Inhibit Endothelial Cell Proliferation**

Alterations in cell shape can have profound effects on proliferation, and EC proliferation is essential for tube extension although not required for initial sprouting (Sholley et al., 1984; Folkman, 1982). The proliferative response of HUVEC in the presence of RMAC11 and AK7 was therefore measured. A decrease in EC proliferation in the presence of RMAC11 or AK7 was seen when the cells were plated on a 2-D matrix of collagen. This was clearly seen with RMAC11

when the assay was performed in the presence of either 20 (data not shown) or 2% FCS (Fig. 9). AK7, induced a significant level of inhibition only when 2% FCS was used in the assays and this level of inhibition was less than that induced by RMAC11. The results in Fig. 9 have been normalized to the no antibody control. When the actual cell numbers were counted in wells containing 30  $\mu$ g/ml QE2E5, AK7, or RMAC11 the increase in cell numbers was 2.6, 2.0, and 1.4-fold, respectively. These results clearly show that the ability of antibodies to inhibit proliferation appears to correlate with enhancement of capillary formation. No inhibition

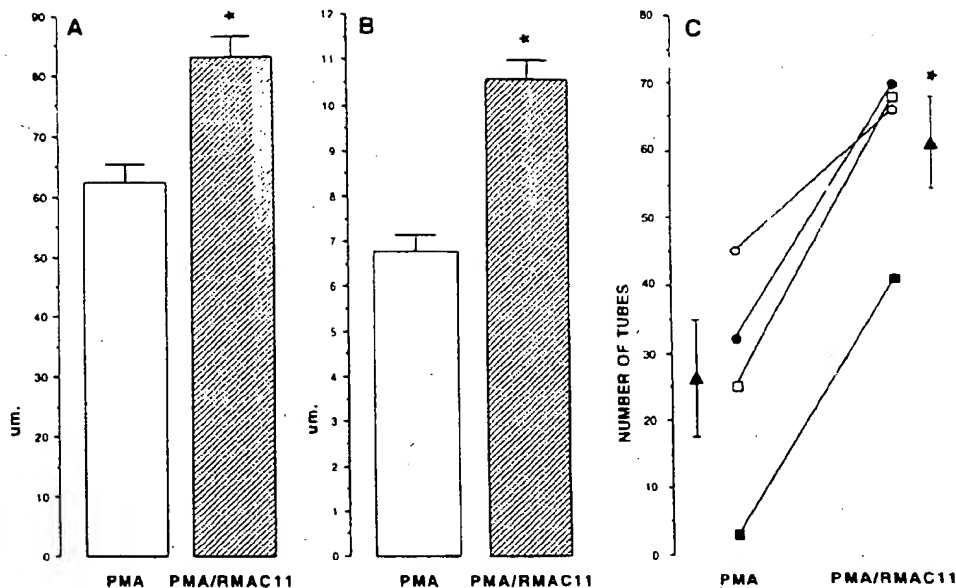


Figure 6. Effect of RMAC11 on tube length ( $\mu\text{m}$  A), width ( $\mu\text{m}$  B), and number (C) per high power field. EC were plated onto collagen gels in the presence of 20 ng/ml PMA. 30  $\mu\text{g/ml}$  of RMAC11 was added to half the wells. Each group contained duplicate wells. 24 h later the wells were photographed (magnification  $\times 264$ ), random fields being taken for each well. From these photographs, the number, length, and width of tubes were calculated. The pooled results from four separate experiments using four different EC lines are shown for tube length and width (mean  $\pm$  SEM). Each experiment showed a similar and significant increase with RMAC11. In C, the tube numbers for each experiment are shown together with the mean  $\pm$  SEM ( $\Delta$ ) for the four experiments. \*  $p < 0.005$  compared to PMA alone.

of proliferation was seen with either antibody when the cells were plated onto fibronectin or gelatin (data not shown).

#### PMA Does Not Alter Expression of $\alpha_2\beta_1$ on Endothelial Cells

One possibility for the effect of anti- $\alpha_2\beta_1$  antibodies on capillary tube formation in collagen gels is that PMA may alter the surface expression of the  $\alpha_2\beta_1$  molecule. To investigate this, HUVEC were plated onto collagen-coated microtiter wells at numbers to give either a confluent monolayer (that is nonproliferating) or a semiconfluent monolayer (that is to give a proliferating population) either in the presence or absence of 20 ng/ml PMA. HUVEC were also plated onto collagen gels in the presence or absence of PMA. 24 h later, the

cells were detached from the microtiter wells with trypsin or extracted from the gels with collagenase. The cells were stained with saturating concentrations of RMAC11 followed by a fluorescein-conjugated sheep anti-mouse Fab<sub>2</sub> antibody. There was no difference in the mean channel fluorescence between confluent and semiconfluent cells either in the presence or absence of PMA, or between tube forming or nonforming cells suggesting that PMA within this time period did not alter the level of surface expression of  $\alpha_2\beta_1$  on HUVEC.

#### Anti-VnR Antibodies Enhance Capillary Formation in Fibrin Gels

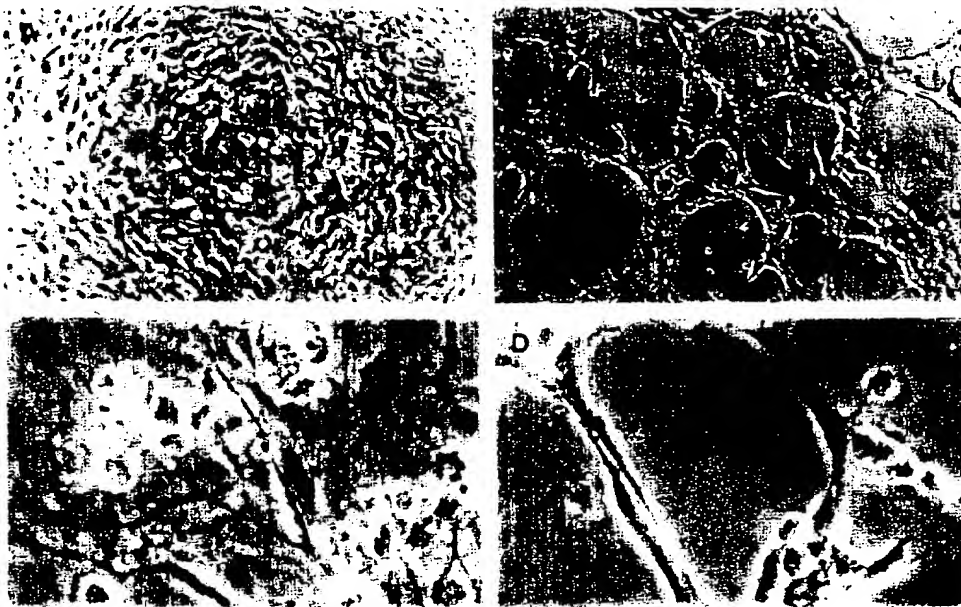
Capillary tube formation also takes place in fibrin gels in the presence of PMA (Montesano et al., 1987). Adhesion of EC to fibrinogen is mediated through another integrin complex, the  $\alpha_v\beta_3$  (or vitronectin receptor, VnR). To determine whether tube formation in fibrin gels is enhanced in the presence of antibodies which limit cell-matrix interactions, we used two antibodies (LM609 or 13C2) which are directed to the  $\alpha_v\beta_3$  complex and known to inhibit EC-fibrinogen adhesion (Cheresh and Spiro, 1987 and unpublished data). Fig. 10 shows that  $\alpha_v\beta_3$  is also involved in the adhesion of HUVEC to fibrin, since anti- $\alpha_v\beta_3$  antibody (LM609) partially inhibits attachment of HUVEC to fibrin-coated plastic (A) but not to collagen (B). Similar results were obtained with 13C2 (data not shown). As seen in Fig. 11, tube formation is enhanced in the presence of LM609 and similarly for 13C2 (data not shown) although the level of tube formation was never as large as that seen in collagen with either RMAC11 or AK7. Functional effects with LM609 were observed with concentrations as low as 3  $\mu\text{g/ml}$ . In the initial 1–2 h on fibrin gels, cells incubated with anti- $\alpha_v\beta_3$  antibody remained rounded and failed to flatten compared to cells

Table 1. Antibody RMAC11 Enhances Capillary Tube Formation in a Dose-dependent Manner

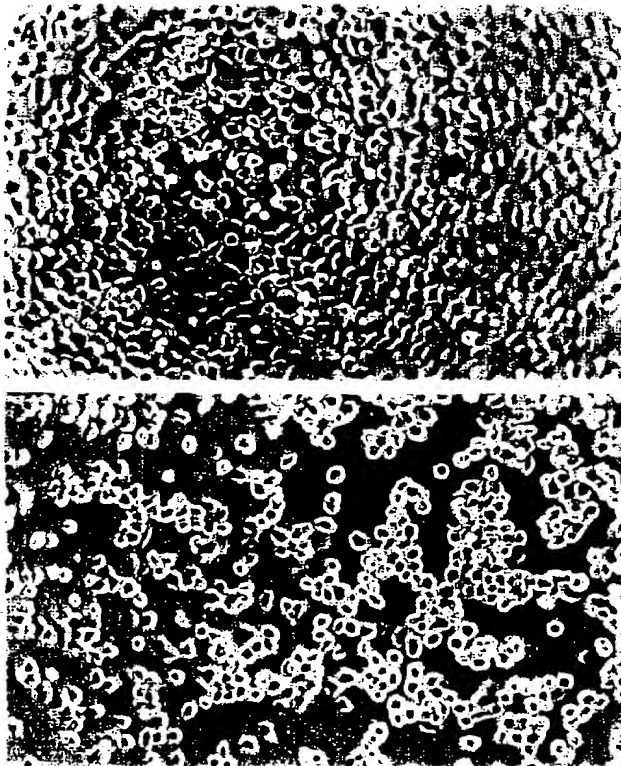
Concentration of RMAC11 ( $\mu\text{g/ml}$ )	Measurement of capillary tubes			Capillary tube formation (+ to +++)
	Number/field	Length ( $\mu\text{m}$ )	Width ( $\mu\text{m}$ )	
0	7	43.9 $\pm$ 2.4	6.1 $\pm$ 0.5	+
0.3	13	46.5 $\pm$ 3.7	7.6 $\pm$ 0.9	++
3	33	52.6 $\pm$ 5.4	11.0 $\pm$ 1.6	+++
10	50	81.8 $\pm$ 8.0	12.5 $\pm$ 0.8	+++
20	87	70.0 $\pm$ 4.5	11.7 $\pm$ 0.9	++++
30	91	75.4 $\pm$ 7.5	11.0 $\pm$ 0.7	++++

EC were plated onto a collagen gel in the presence of 20 ng/ml PMA together with varying concentrations of RMAC11. Measurement of the number, length, and width of tubes was made from high power photographs taken at random from each well, the measurements for length and width are given as mean  $\pm$  SEM. The extent of capillary tube formation was also assessed and is given as + to ++++ based on the length, width, and number of capillary tubes. The results are shown for one experiment which was similar to three performed, each with a different EC line.

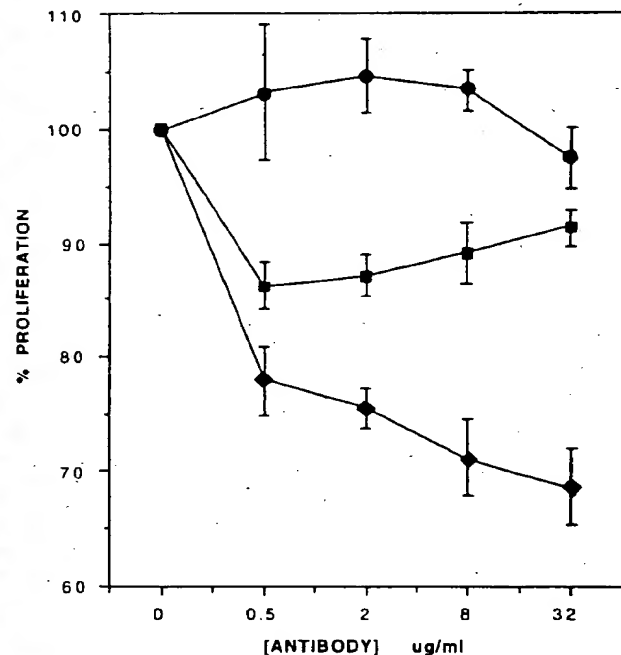




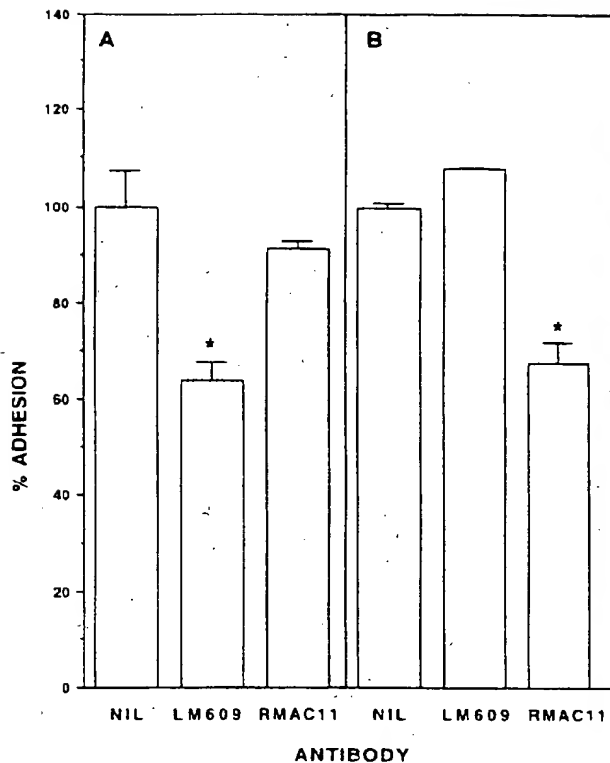
**Figure 7.** Anti-integrin antibodies promote tube formation in rigid collagen gels. EC were plated onto collagen which had been allowed to gel for 1 h at 37°C giving a more rigid gel than that obtained after our normal gelling time of 10–20 min. C and D are high powered views of A and B, respectively. 20 ng/ml of PMA was added to both groups, 30 µg/ml of RMAC11 was added to B. Tube formation was assessed after 24 h. A and B magnification ×80; C and D magnification ×310. Each is a representative field of one well of duplicate wells set up for each group. The experiment has been performed at least six times using different EC lines and using either RMAC11 or AK7 with similar results being obtained.



**Figure 8.** Anti-integrin antibodies maintain EC in a rounded morphology and prevent cell spreading. EC were plated onto collagen I gels with 20 ng/ml PMA either in the presence of 30 µg/ml QE2E5 (A) or 30 µg/ml RMAC11 (B). The cells were viewed at 1, 2, 3, 4, and 6 h after plating. These photographs were taken at 2 h after plating (magnification ×110). Each shows a representative field of one well from duplicate wells set up for each group. The experiment has been performed on at least four separate EC lines with similar results.



**Figure 9.** Anti- $\alpha_2\beta_1$  antibodies inhibit EC proliferation. EC were plated onto collagen I-coated microtitre wells at  $5 \times 10^3$  cells/well in HUVEC medium containing 2% FCS either with QE2E5 (●), RMAC11 (◆), or AK7 (■) at various concentrations. The cells were incubated for 3 d at 37°C, washed, fixed, stained with methylene blue, and the dye solubilized with ethanol. Absorbance was read at 630 nm. The OD630 of wells with no antibody was taken to give 100% proliferation. All other groups were normalized to this. The results show the mean  $\pm$  SEM of four experiments where each point in each experiment was performed with six replicates. Groups containing RMAC11 and AK7 were significantly different ( $p < 0.0001$ ) from groups containing QE2E5 (ANOVA test for significance).

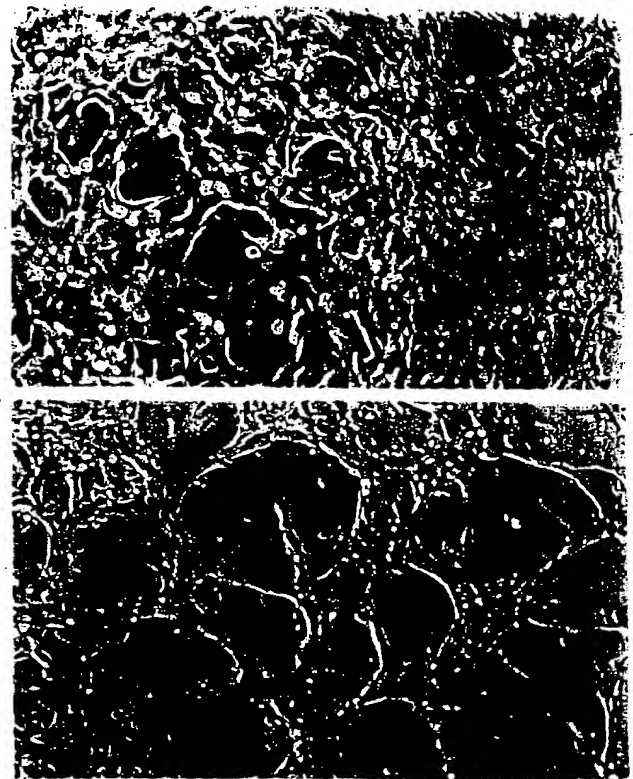


**Figure 10.** Anti- $\alpha_2\beta_1$  antibody partially inhibits EC adhesion to fibrin (A) but not to collagen (B). Fibrin-coated microtiter wells were formed by thrombin cleavage of fibrinogen followed by two washes in medium containing FCS. Antibodies as indicated were added at a final concentration of 30  $\mu\text{g/ml}$  to either fibrin (A) or collagen (B)-coated wells. The plates were incubated at 37°C for 1 h, washed, and the number of attached cells assayed as given for Fig. 1. The results show the mean of triplicate wells for each group of one experiment representative of three experiments.  $p < 0.01$  compared to no antibody group on either matrix.

without antibody. Sections analyzed by light microscopy showed details similar to that observed in collagen gels. With PMA alone, small lumen formed by intracellular vacuolization were seen. In the presence of LM609 or 13C2 there was extensive cell-cell interactions and lumina were formed between cells. Fig. 12 shows that in the presence of anti- $\alpha_2\beta_1$  antibody, the length, width, and the number of tubes were increased over that seen with PMA alone. Analysis of thin sections showed that with PMA alone, the majority of lumen were formed from single intracellular lumen although lumina formed from two cells were occasionally seen. In the presence of anti- $\alpha_2\beta_1$  antibody, the majority of lumina (>70%) were formed from three cells with some lumina formed from four or five cells. The enhancement of tube formation seen with  $\alpha_2\beta_1$  antibodies and with anti- $\alpha_2\beta_1$  antibodies was specific for the matrix used;  $\alpha_2\beta_1$  antibodies had no effect in fibrin gels and  $\alpha_2\beta_1$  antibody had no effect in collagen gels (data not shown).

## Discussion

The central finding of this study is that anti-integrin antibodies



**Figure 11.** Anti- $\alpha_2\beta_1$  antibody promotes capillary formation in fibrin gels.  $6.4 \times 10^4$  EC/well were plated onto fibrin gels in the presence of 20 ng/ml PMA. The anti- $\alpha_2\beta_1$  antibody LM609 at a final concentration of 30  $\mu\text{g/ml}$  was added to group B. Cells were incubated overnight. A representative field of one well of duplicate wells in each group is shown (magnification  $\times 110$ ). The experiment has been performed at least five times using a different EC line for each experiment, all giving similar results.

are able to enhance the formation of capillary tubes in vitro. Functional monoclonal antibodies directed to the major integrin receptors for the tube-permissive matrices of collagen and fibrin enhanced capillary tube formation increasing the number, length, as well as width. Ingber and Folkman (Ingber and Folkman, 1989a,b; Ingber, 1990, 1991a,b) propose that changes in adhesivity (e.g., by altering the density of ECM molecules), and therefore in the ability of the matrix to resist cell tension, may result in alteration in cell function such as proliferation and differentiation. Thus the mechanical forces between cells and their environment will govern their behavior. Our results showing that anti-integrin antibodies, which block adhesion, enhance tube formation suggest that angiogenesis may also be regulated by the adhesivity of EC for the matrix. The balance between cell and matrix adhesivity will clearly be important in determining the function of endothelial cells.

In the presence of antibody (anti- $\alpha_2\beta_1$  and anti- $\alpha_2\beta_1$ ), the first change observed was that the EC remained rounded, became less adhesive, and failed to spread when plated onto gels or 2-D matrices rather than adopting their normal cobblestone, flattened morphology. It is known that alteration in cell shape can have profound effects on the function of many

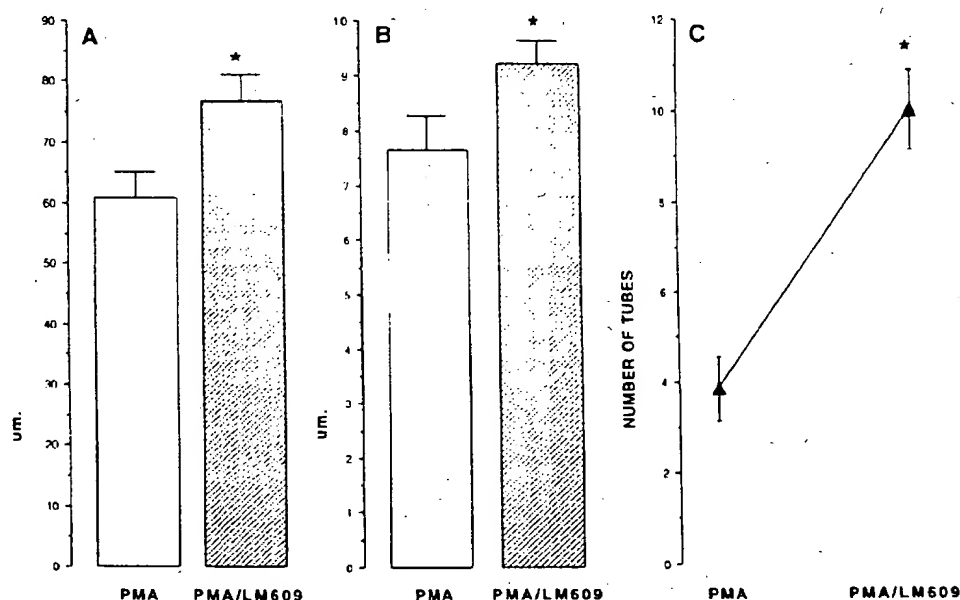


Figure 12. Effect of LM609 on tube length ( $\mu\text{m}$ , A), width ( $\mu\text{m}$ , B), and number (C) per high power field. EC were plated onto fibrin gels in the presence of 20 ng/ml PMA, and 30  $\mu\text{g/ml}$  LM609 was added to half the wells. 24 h later the wells were photographed (magnification  $\times 264$ ), random fields being taken for each well. The number, length, and width of tubes were measured from these photographs. The pooled results from two separate experiments are given for the length, width, and number. \*  $p < 0.05$  compared to PMA alone.

cells resulting in changes to cell proliferation and differentiation (Folkman and Moscona, 1978). Gospodarowitz has reported that EC must become attached and flattened in order to proliferate and that those kept rounded in suspension fail to divide (Gospodarowitz et al., 1978). Furthermore, there is a direct correlation between growth inhibition and decreases in cell extension (Ingber et al., 1987). DNA, RNA, and protein synthesis in anchorage dependent fibroblasts is also inhibited if these cells are maintained in suspension (Ben-Ze'ev, 1980). Differentiation and expression of genes which reflect a more differentiated state of hepatocytes, chondrocytes, fibroblasts, and endothelial cells are linked to cell shape and actin reorganization (Aggeler et al., 1984; DiPersio et al., 1991; Mallein-Gerin et al., 1991; Ingber and Folkman, 1989a,b; Ben-Ze'ev et al., 1988; Glowacki et al., 1983; Unemori and Werb, 1986; Werb et al., 1986). All these studies suggest that the mechanical interaction of cells with the ECM can regulate cell function. Since integrins interconnect the ECM with the cyto-skeleton, they are likely to be involved in the transmission of signals between the cell and its ECM. The biochemical signals (termed mechanotransducers) which are generated as a result of mechanical forces or integrin activation are unknown at present but phosphorylation (Kornberg et al., 1991), activation of the  $\text{Na}^+/\text{H}^+$  exchanger (Ingber, 1990),  $\text{Ca}^{++}$  mobilization, and adenylate cyclase (for review see Watson, 1991; Ingber, 1991b) have been implicated.

In the studies reported here, the decrease in the adhesion and change in cell shape of EC with anti-integrin antibodies, resulted in an inhibition in proliferation and in a promotion of differentiation as measured by capillary tube formation. These alterations in cell function were matrix specific, that is anti- $\alpha_2\beta_1$  antibodies inhibited EC proliferation and enhanced cell differentiation only on a collagen but not on a fibrin gel, while anti- $\alpha_5\beta_1$  showed effects on fibrin but not on collagen gels. Thus, the effect of anti-integrin antibodies on angiogenesis is ligand dependent. Furthermore, the antibody mediated effects are time dependent. No enhancement

of capillary formation was observed when the antibodies were added more than 2 h after cell plating, paralleling the time dependency seen with the antibodies on EC adhesion and cell shape.

EM revealed striking qualitative changes in tubes with anti-integrin antibodies. In the absence of RMAC11, the majority of lumina were formed within single cells in a manner reported by Folkman and Haudenschild (1980). However, with RMAC11, the majority of lumina were formed from multiple cells with clear cellular borders between cells making up the vessel (Fig. 3 B). Using fibrin gels and anti- $\alpha_2\beta_1$  antibodies, lumina were also formed from multiple cells. Thus anti-integrin antibodies not only alter the degree of tube formation taking place but also influence the phenotype of tubes. The relationship between intracellular and intercellular lumina is not known at present but one possibility is that intercellular lumen form from coalescence of intracellular vacuole-like structures with the plasma membrane and that these structures define stages in tube formation. Clearly, our in vitro model of angiogenesis may allow a more detailed examination of the stages involved in capillary formation.

The enhancement of capillary tube formation with anti-integrin antibodies was dependent on the presence of PMA since the antibodies alone had no effect. Thus, one signal for tube formation is likely to be protein kinase C-dependent. Indeed activators of protein kinase C inhibit the proliferation of EC in response to mitogens (Doctrow and Folkman, 1987). One possibility for our results is that the antibodies enhance PMA-mediated signals. This, however, is unlikely since the antibodies do not enhance tube formation on an inappropriate matrix. That is, anti- $\alpha_2\beta_1$  antibody had no effect on fibrin gels, and anti- $\alpha_5\beta_1$  antibody had no effect on collagen gels even though tube formation can take place on these matrices. This data therefore suggests that the antibodies do not directly signal the cell to undergo tube formation. This is further supported by the fact that pretreatment of HUVEC with the antibodies and removal by washing does not result in enhanced tube formation.



No capillary tube formation is induced on collagen gels that are too rigid even in the presence of PMA. The cells form a confluent monolayer and little or no invasion into the matrix takes place suggesting that EC differentiation was inhibited by the rigid ECM. However, anti- $\alpha_3\beta_1$  antibody on rigid gels in the presence of PMA did induce EC invasion into the gel and subsequent tube formation. The cells in the presence of RMAC11 were more rounded than with control antibody and failed to spread and flatten. A consequence of changes in cell shape, adhesion, and signaling may be an alteration in the level of matrix degrading enzymes such as collagenase. However, we observed no change in the total collagenase produced by the EC in the presence of RMAC11. One possibility to explain these results is that there is an alteration in the site of collagenase release rather than an alteration in the overall level of production. Enzyme redistribution has been demonstrated for urokinase plasminogen activator (uPA) after anti-fibronectin antibody binding to rabbit fibroblasts (Werb et al., 1989). Thus, as a consequence of antibody-integrin binding (and perhaps integrin redistribution and change in cell shape) collagenase may be redirected to specific localized areas resulting in enhanced cell motility and gel invasion. In addition, since activation of collagenase can occur via cleavage by uPA measurement of uPA or its receptor may indicate altered enzyme activity (Mignatti et al., 1991).

Another possibility to explain the enhancement of tube formation by the antibodies is that the binding of the anti-integrin antibodies to their antigen may simply limit the number of receptors available for cell-matrix interactions thereby resulting in enhanced motility of the cell within the matrix. Indeed, an alteration in the adhesivity of EC for different matrices via changes in the level of expression of the integrins,  $\alpha_1\beta_1$  and  $\alpha_v\beta_3$ , can be achieved by cytokines such as tumor necrosis factor and interferon- $\gamma$  (Defilippi et al., 1991a,b). The anti-integrin antibodies may also limit the number of focal contacts which can form an important function mediated through the  $\beta$  subunit (Solowska et al., 1989), reducing adhesion, and enhancing the lateral mobility of the integrins within the cell membrane. This lateral mobility of integrins is known to be important for cell movement (Duband et al., 1988). Alternately, the limitation in the available number of functional  $\alpha_3\beta_1$  or  $\alpha_v\beta_3$  molecules may redirect the cell to use other integrin molecules. It is interesting to note that RMAC11 or AK7 cannot totally inhibit HUVEC binding to collagen. Anti- $\beta_1$  antibodies further inhibit this adhesion suggesting that other  $\beta_1$  integrins are involved.  $\alpha_1\beta_1$  and  $\alpha_3\beta_1$  are able to mediate adhesion to collagen at least in some cells (Defilippi et al., 1991a; Hemler et al., 1990). Whether these integrins can participate in angiogenesis remains to be determined. The use of alternate matrix receptors may induce a different set of signals which in our system is manifested in enhanced capillary formation. Using chimeric constructs transfected into RD cells, Chan et al. (Chan et al., 1992) have shown that the cytoplasmic domains of the integrin receptors can mediate different signals irrespective of the ligand-binding event.

Angiogenesis is clearly a complex event that can be regulated at multiple levels. In this paper we have demonstrated that the extent of capillary tube formation can be enhanced by the use of anti-integrin antibodies specific for a given receptor-ligand system which inhibit cell-matrix adhesion.

These antibodies have profound effects on cell shape, adhesion, proliferation, and subsequent cell differentiation. A corollary of the work presented here suggests that anti-integrin antibodies which promote cell adhesion to the ECM will actually inhibit in vitro angiogenesis. Thus regulation of angiogenesis may be mediated through alteration in the matrix (as has been shown previously), or as our results suggest, by alteration in the function of matrix-adhesion receptors on the EC.

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**IP AUSTRALIA**

**PATENT OPPOSITION**

**Ludwig Institute for Cancer Res.**

**And**

**Human Genome Sciences Inc**

**Bundle 4 of 4**

**Australian Patent Appln 696764 (73941/94)**

**“Vascular Endothelial Growth Factor 2”**

**Evidence in Answer**

**(1) Gary Cox’s Statutory Declaration Annexures GBC-12 to GBC-23.**

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COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

IN THE MATTER OF: Australian

Patent Application 696764

(73941/94). In the name of:

Human Genome Sciences Inc.

- and -

IN THE MATTER OF: Opposition

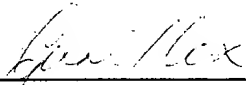
thereto by Ludwig Institute for Cancer

Research, under Section 59 of the

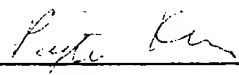
Patents Act.

Annexure GBC-12

This is **Annexure GBC-12** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
\_\_\_\_\_  
**Gary Baxter Cox**

WITNESS:

  
\_\_\_\_\_  
Patent Attorney

PEYTEE K HALL



US006130071A

**United States Patent** [19]

Alitalo et al.

[11] **Patent Number:** 6,130,071[45] **Date of Patent:** Oct. 10, 2000

- [54] **VASCULAR ENDOTHELIAL GROWTH FACTOR C (VEGF-C) ΔCYS<sub>156</sub> PROTEIN AND GENE, AND USES THEREOF**
- [75] Inventors: Kari Alitalo, Espoo; Vladimir Joukov, Helsinki, both of Finland
- [73] Assignee: Helsinki University Licensing, Ltd., Helsinki, Finland
- [21] Appl. No.: 08/795,430
- [22] Filed: Feb. 5, 1997
- [51] Int. Cl.<sup>7</sup> ..... C07K 14/475; C12N 15/18
- [52] U.S. Cl. .... 435/69.4; 530/350; 530/399; 435/320.1; 435/325; 536/23.51; 514/2; 514/12
- [58] Field of Search ..... 530/399, 350; 435/69.4, 320.1, 325; 536/23.51

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**Primary Examiner**—John Ulm**Assistant Examiner**—Eileen B. O'Hara**Attorney, Agent, or Firm**—Marshall, O'Toole, Gerstein, Murray & Borun

[57]

**ABSTRACT**

Provided are purified and isolated VEGF-C cysteine deletion variants that bind to Flt4 receptor tyrosine kinase (VEGFR-3) but demonstrate reduced binding (relative to VEGF-C) to kdr receptor tyrosine kinase (VEGFR-2); polynucleotides encoding the polypeptide; vectors and host cells that embody the polynucleotides; pharmaceutical compositions and diagnostic reagents comprising the polypeptides; and methods of making and using the foregoing.

**29 Claims, 20 Drawing Sheets**

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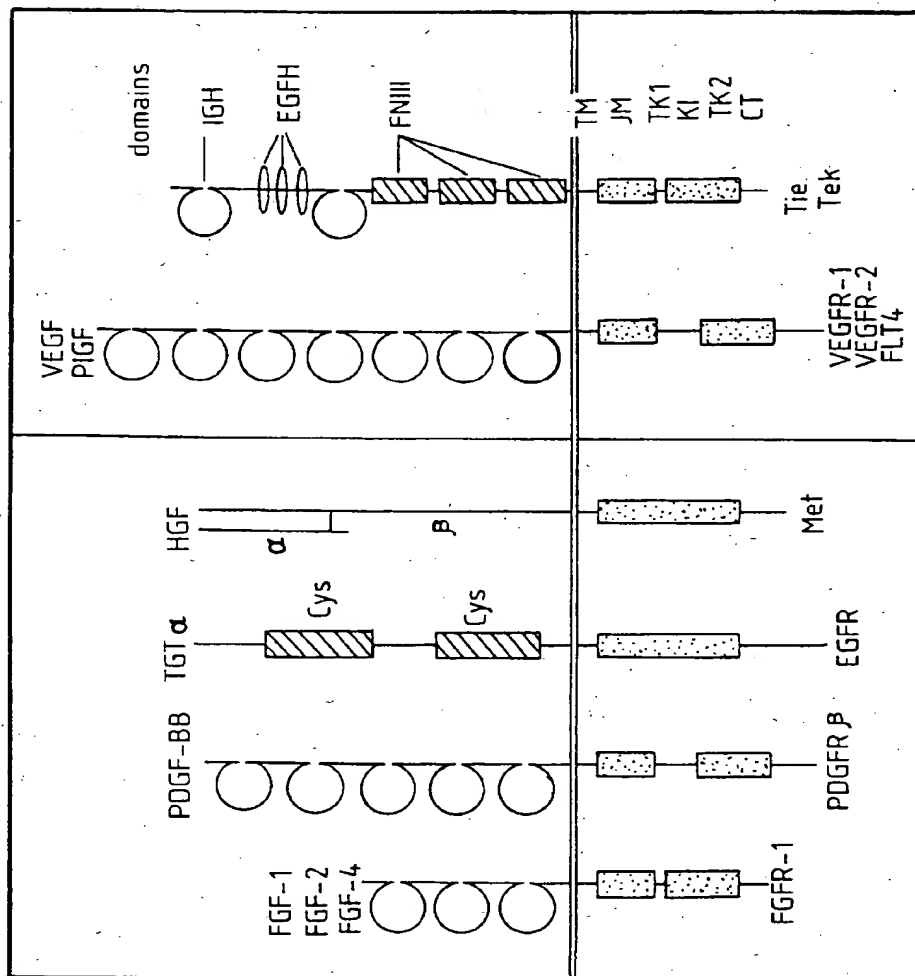
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FIGURE 1



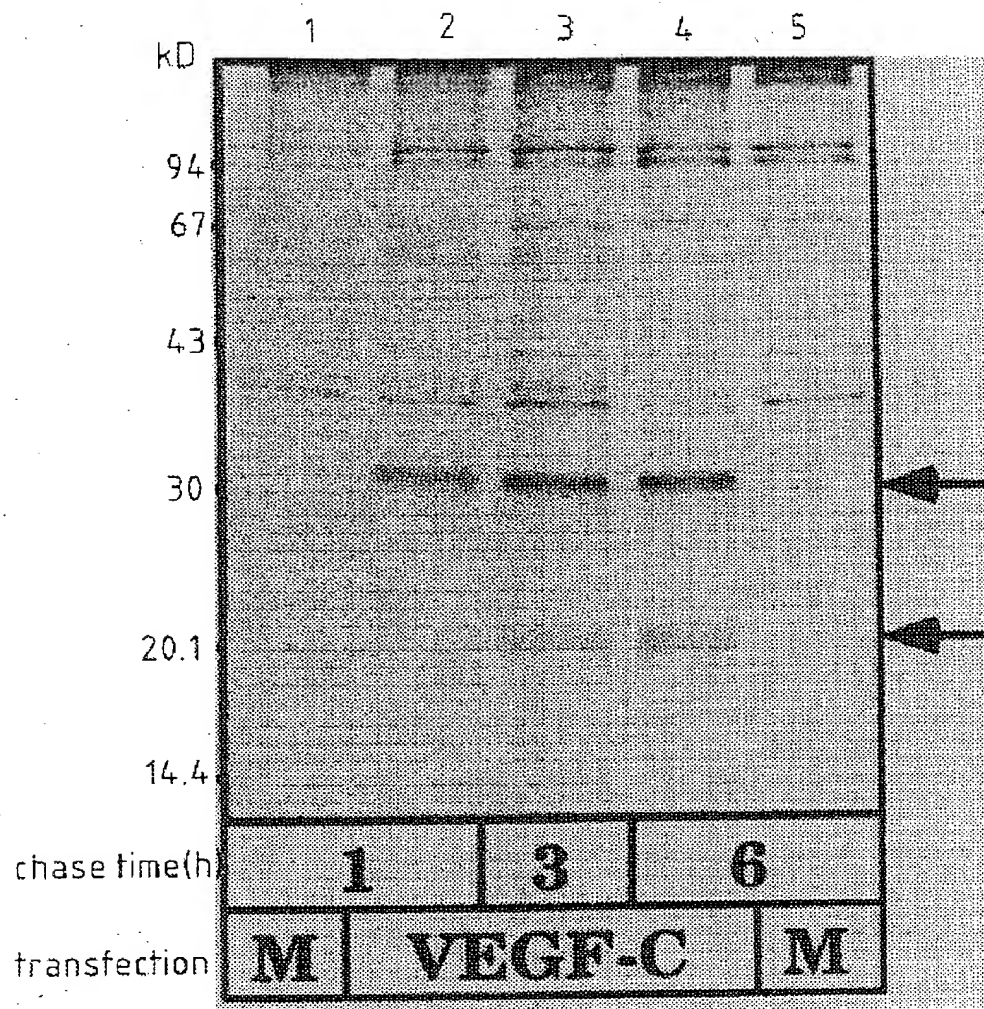
1	PDGF-A	.....	.....	.....	50
	PDGF-B	.....	.....	.....	MRTLACLLL
	PIGF-1	.....	.....	.....	MNRCWA.LFL
	VEGF165	.....	.....	.....	.....
	VEGF-B167	.....	.....	.....	.....
	VEGF-C	MHLGGFFSVA	CSLLAAALLP	GPREAPAAA	AFESGLDLS
					AEPDAGENTA
51	PDGF-A	LGGYLAVHL	AEEAIEPREV	IERLARSQIH	SIRDQLRLE
	PDGF-B	SLCCYLRLVS	AEGDPIPEEL	YEMLSHSIR	SFDDQLRLLH
	PIGF-1	.....	.....MPVM	RLFPC..FLQ	LLAGLAL...
	VEGF165	.....	.....	NFLLS..WVH	WSLALLLYLH
	VEGF-B167	.....	.....	SPLLR..RL	LAALLQLAPA
	VEGF-C	YASKDLEEQL	RSVSSVDELM	TVLYPEYWM	YKQLRKGGW
					QHNREQANLN
101	PDGF-A	DTSLRAHGVH	ATKHVPEKRP	LPIRRKRSL	.....
	PDGF-B	ELDLNMTSRH	SGGELES...	LARGRRSLG	SULTIAEPAMI
	PIGF-1	.....	ALSAG	NGSSEVEVP	FQE.VWGR..
	VEGF165	.....	PMAEG	GGQNHHEVVK	FMD.VYQR..
	VEGF-B167	.....	D	APGHQRKVVS	WID.VYTR..
	VEGF-C	SRTEETIKFA	AAHYNTEILK	SIDNEWK..	.....
					TOCMPREVC
151	PDGF-A	EIPRSQVDPT	SANFLIWPPC	VEVKRCTGCC	NTSSVKCQPS
	PDGF-B	EISRRLLDRT	NANFLVWPPC	VEVQRCSGCC	NNRNVCQCRPT
	PIGF-1	DVWSEYPSEV	..EHMFSPSC	VSLLRCTGCC	GDENLHCVPV
	VEGF165	DIFQEYPDEI	..EYIFKPS	VPLMRCGGCC	NDEGLECVPT
	VEGF-B167	PLTVELMGTV	..AKQLVPSC	VTVQRCGGCC	PDDGLECVPT
	VEGF-C	DVGKEFGVAT	..NTFFKPPC	VSVMRCGGCC	NSEGLQCMNT
					STSLSKTLF
200	PDGF-A	EIPRSQVDPT	SANFLIWPPC	VEVKRCTGCC	NTSSVKCQPS
	PDGF-B	EISRRLLDRT	NANFLVWPPC	VEVQRCSGCC	NNRNVCQCRPT
	PIGF-1	DVWSEYPSEV	..EHMFSPSC	VSLLRCTGCC	GDENLHCVPV
	VEGF165	DIFQEYPDEI	..EYIFKPS	VPLMRCGGCC	NDEGLECVPT
	VEGF-B167	PLTVELMGTV	..AKQLVPSC	VTVQRCGGCC	PDDGLECVPT
	VEGF-C	DVGKEFGVAT	..NTFFKPPC	VSVMRCGGCC	NSEGLQCMNT
					STSLSKTLF

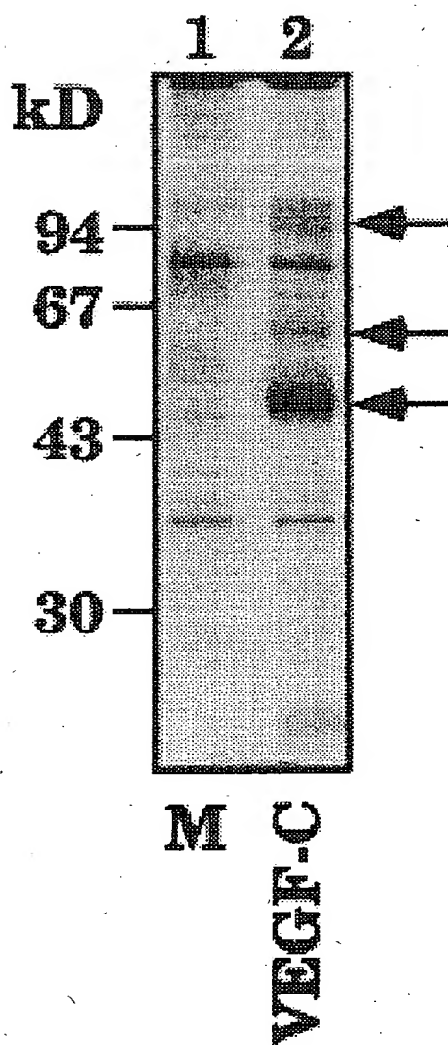
FIGURE 2A

## FIGURE 2B

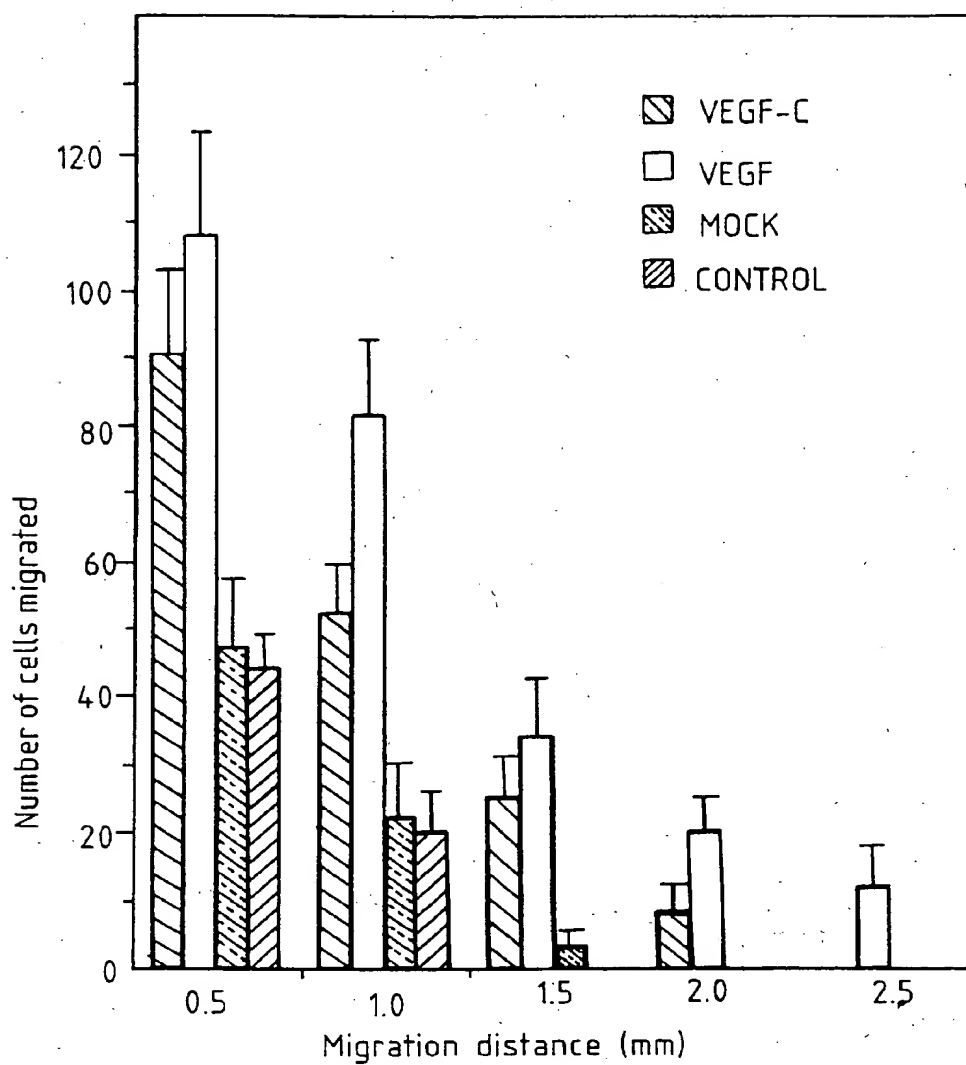
351	400
PDGF-A	.....
PDGF-B	.....
PlGF-1	.....
VEGF165	.....
VEGF-B167	.....
VEGF-C	ENTCQCVCCKR TCPRNQPLNP GKACECTES PQKCLLKGGK FHHQTCSCYR
401	434
PDGF-A	.....
PDGF-B	.....
PlGF-1	.....
VEGF165	.....
VEGF-B167	.....
VEGF-C	RPCTNRQKAC EPGFSYSEEV CRCVPSYWKR PQMS

FIGURE 2C

**FIGURE 3A**

**FIGURE 3B**

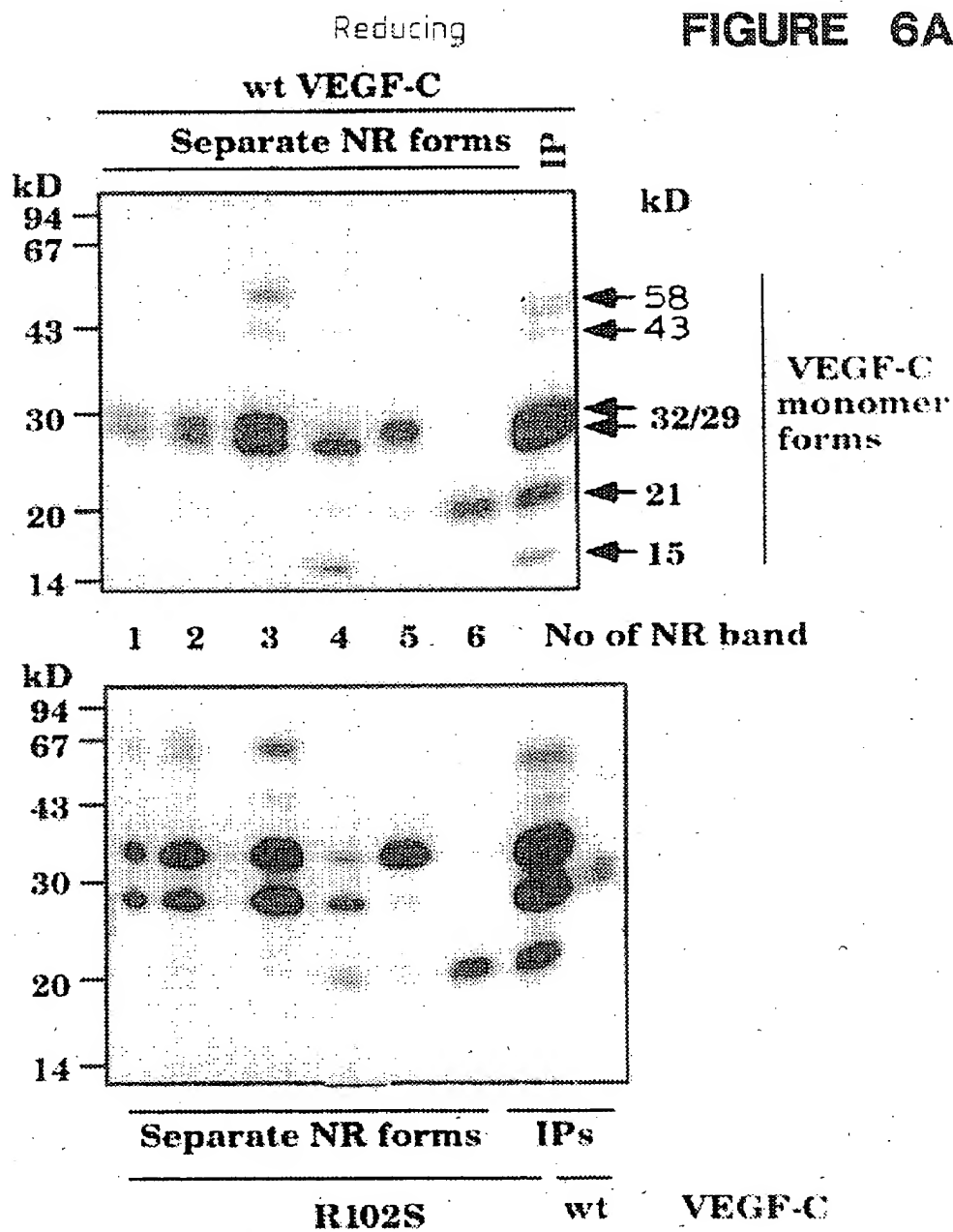


**FIGURE 4**

## VEGF-C alignment

	1				50
Hum	HMLLGFFSVA	CSLLAAALLP	GPREAPAAAA	AFESGLDLSL	AEPDAGEATA
Mou	MHLLCFLSLA	CSLLAAALIP	SPREAPATVA	AFESGLGFSE	AEPDGGGEVKA
Qua	MHLLLEMLSLG	CCLAAGAVLL	GPRQPPVA.A	AYESGHGYE	EEPGAGEPKA
	51				100
Hum	YASKDLEEQL	RSVSSVDELM	TVLYPEYWK	YKQQLRKGGW	QHNREQANLN
Mou	FEGKDLEEQL	RSVSSVDELM	SVLYPDYWK	YKQQLRKGGW	Q....OPTLN
Qua	HASKDLEEQL	RSVSSVDELM	TVLYPEYWK	FKQQLRKGGW	QHNREHSSSD
	101				150
Hum	SRTEETIKFA	AAHYNTEILK	SIDNEWRTQ	CMPREVCIDV	GKEFGVATNT
Mou	TRTGDSVKFA	AAHYNTEILK	SIDNEWRTQ	CMPREVCIDV	GKEFGAATNT
Qua	TRSDDSLKFA	AAHYNAEILK	SIDTEWRTQ	CMPREVCIDL	GKEFGATTNT
	151				200
Hum	FFKPPCVSVY	RCGGCCNSEG	LQCMNTSTSY	LSKTLFEITV	PLSQGPKPVT
Mou	FFKPPCVSVY	RCGGCCNSEG	LQCMNTSTGY	LSKTLFEITV	PLSQGPKPVT
Qua	FFKPPCVSIY	RCGGCCNSEG	LQCMNISTNY	ISKTLFEITV	PLSHGPKPVT
	201				250
Hum	ISFANETSCR	CMSKLDVYRQ	VHSIIRSLP	ATLPQCOAAN	KTCPTNYMWN
Mou	ISFANETSCR	CMSKLDVYRQ	VHSIIRSLP	ATLPQCOAAN	KTCPTNYVWN
Qua	VSFANETSCR	CMSKLDVYRQ	VHSIIRSLP	ATQTQCHVAN	KTCPKNHVWN
	251				300
Hum	NHICRCLAQE	DFMPSSDAGD	DSTDGFHDIC	GPNKELDEET	CQCVCRAGLR
Mou	NYMCRCCLAQ	DFIFYSNVED	DSTNGFHDVC	GPNKELDEET	CQCVCCKGGLR
Qua	NQICRCLAQH	DFGFSHLGD	SDTSEGFHIC	GPNKELDEET	CQCVCCKGGVR
	301				350
Hum	PASCGPHKEL	DRNSCQCVC	NKLFPSQCGA	NREFDENTCQ	CVCKRTCPRN
Mou	PSSCGPHKEL	DRDSCQCVC	NKLFPSQCGA	NREFDENTCQ	CVCKRTCPRN
Qua	PISCGPHKEL	DRASCQCMCK	NKLLPSSCGP	NKEFDEEKCQ	CVCKKTCPKH
	351				400
Hum	QPLNPGKAC	ECTESPQKCL	LKGKKFHHQT	CSCYRRPCTN	RQKACEPGFS
Mou	QPLNPGKAC	ECTENTQKCF	LKGKKFHHQT	CSCYRRPCAN	RLKHCDPGLS
Qua	HPLNPAKCIC	ECTESPNKCF	LKGKRFHHQT	CSCYRPPCTV	RTKRCDAFL
	401		420		
Hum	YSEEVCRGVP	SYWKRPMQMS*			
Mou	PSEEVCRGVP	SYWKRPHLN.			
Qua	LAEEVCRGVR	TSWKRPLMN*			

FIGURE 5

**FIGURE 6C**

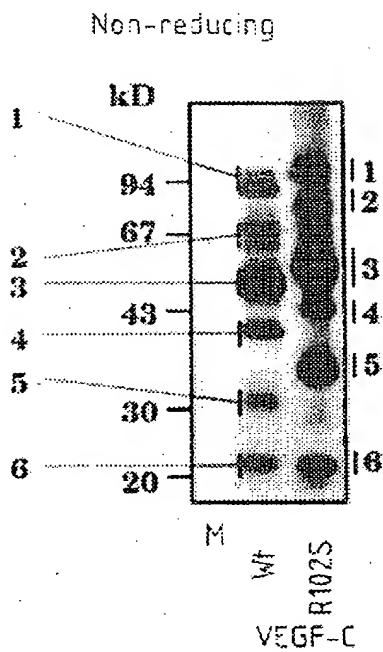


FIGURA 6B

FIGURA 7A

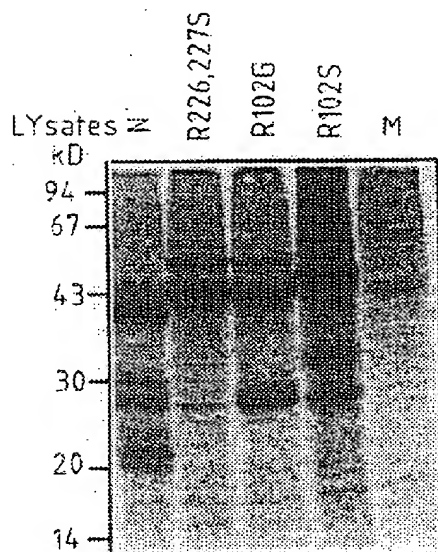
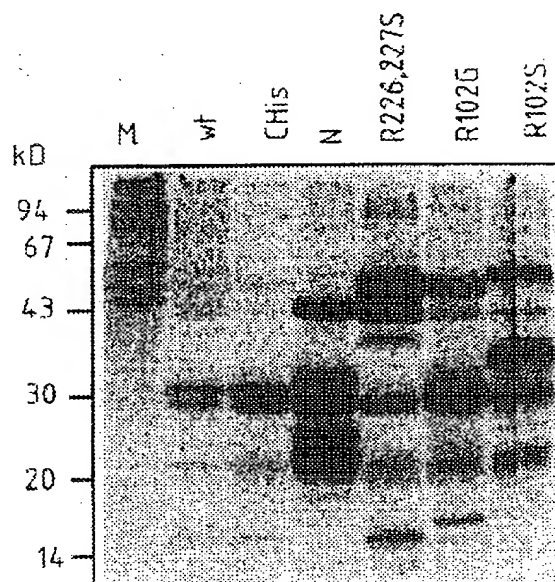


FIGURA 7B

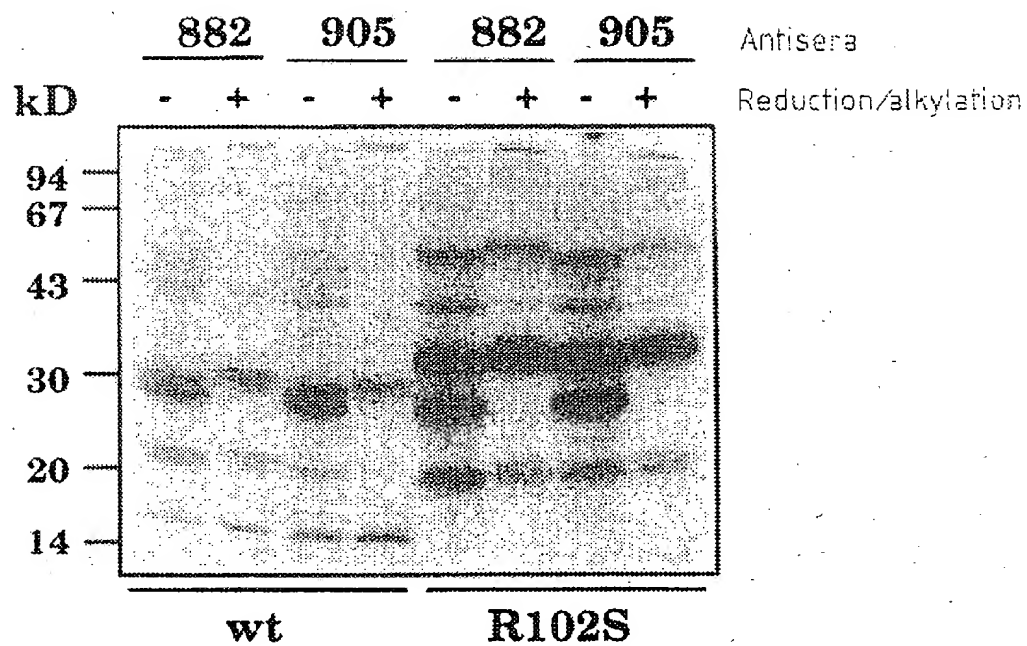


FIGURE 8A

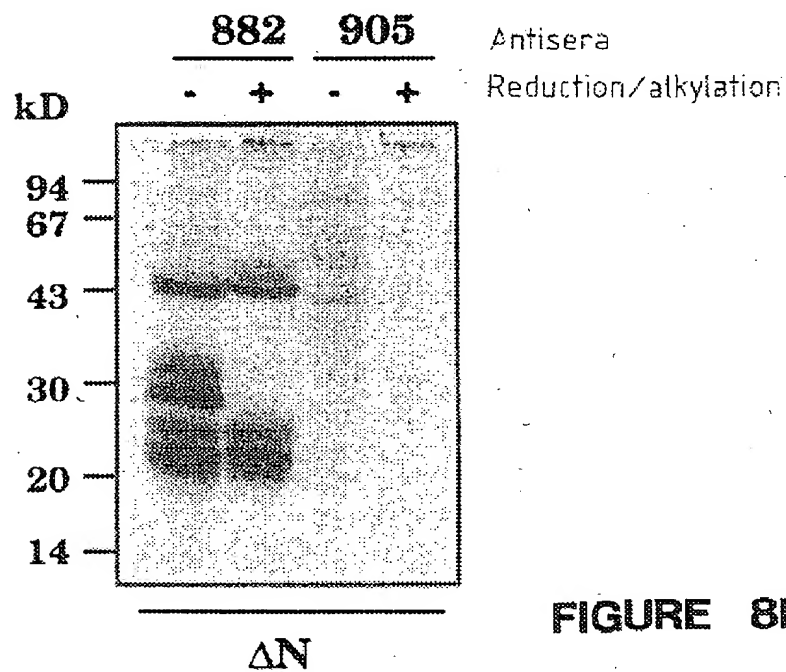


FIGURE 8B

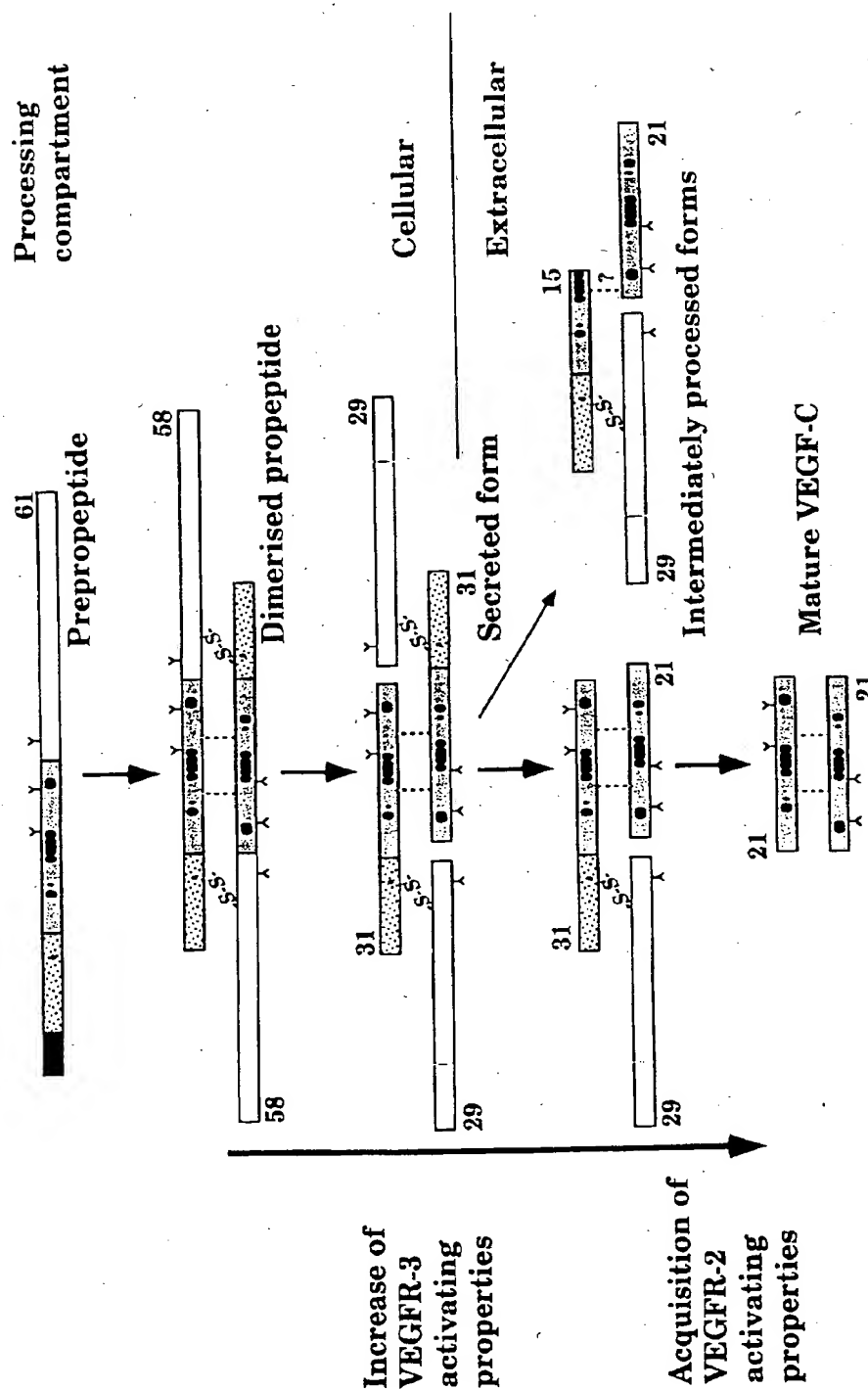
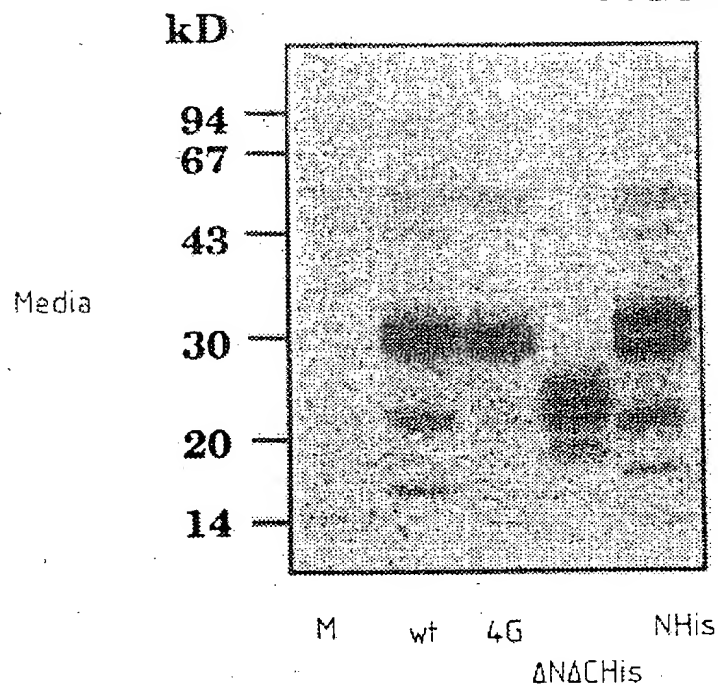
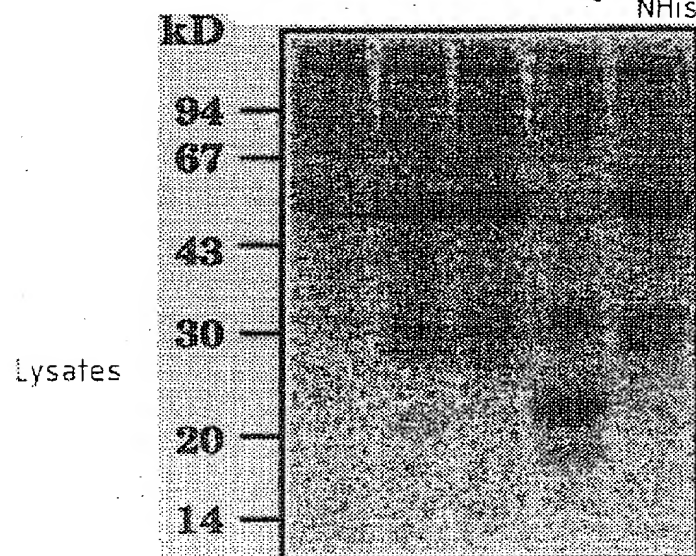


FIGURE 9

## VEGF homology

### BR3P homology

[illegible]

**FIGURE 11A****FIGURE 11B**



**FIGURE 12**

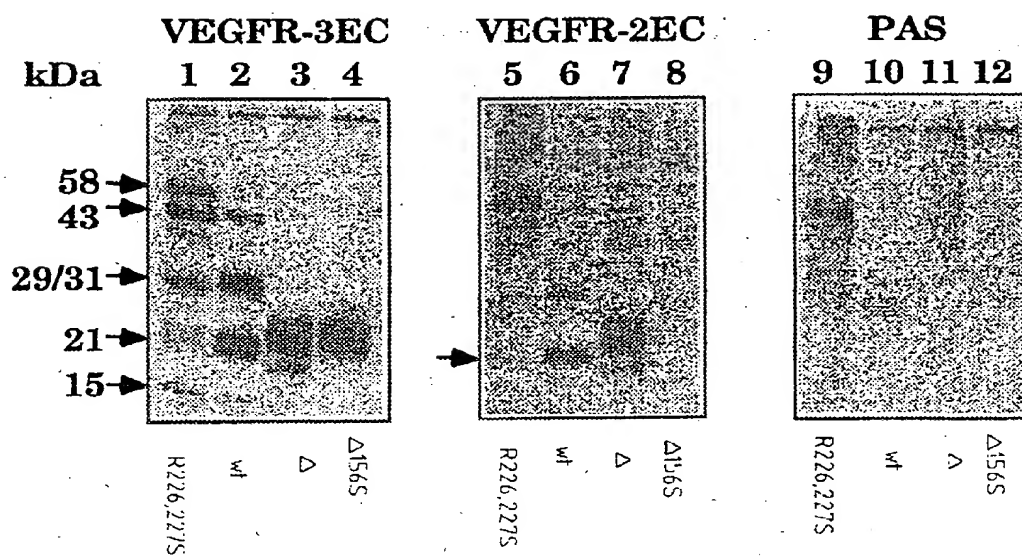
HUMAN				
Exon length	Donor site	Intron length	Acceptor site	
.....	G...E...A...T(49)	.....	A...Y...A...S.	
E1.....	GGC.GAG.GCC.ACG.gtaggtctgct>10.kb..TTTCTTTGACAG.GCT.TAT.GCA.AGC			
.....	E...I...L...K(116)	.....	S...I...D...N.	
E2.214.bp.	GAG.ATC.TTG.AAA.Agtaagtatgg...1.6.kb...atgacttgacagGT.ATT.GAT.AAT			
.....	L...S...K...T(180)	.....	L...F...E...I.	
E3.191.bp.	CTC.AGC.AAG.ACG.gtgggtattgt.....9.kb.cccttctttag.TTA.TTT.GAA.ATT			
.....	T...L...P...Q(231)	.....	C...Q...A...A.	
E4.152.bp.	ACA.CTA.CCA.CAgtagtatgaattaaa>10.kb..ttcttccaaagG.TGT.CAG.GCA.GCG			
.....	A...G...D... (266)	.....	D...S...T...D.	
E5.107.bp.	GCT.GGA.GAT.Ggtagcagaatg.....301.bp...ctatttgcttagAC.TCA.ACA.GAT			
.....	Q...T...C...S(378)	.....	C...Y...R...R.	
E6.334.bp.	CAA.ACA.TGC.AGgtaagagatcc.....>10.kb..tggtctccttagC.TGT.TAC.AGA.CGG			
.....	Q...M...S(419) Stop	.....		
E7.(501).bp.	CAA.ATG.AGC.TAA.GTATGACTGTT...ATTGTATTAT			

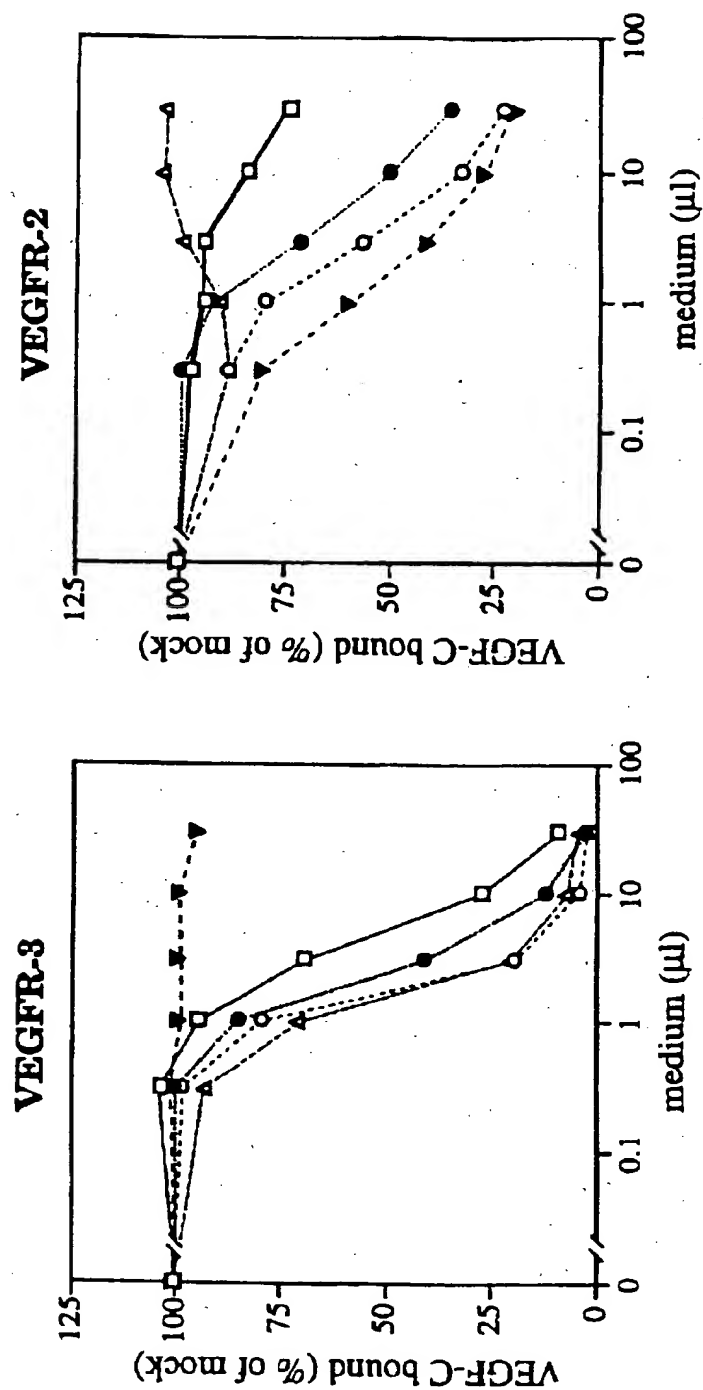
FIGURE 13A

MOUSE Exon length	Donor site	Intron length	Acceptor site
.....G...E...V...K(49).....			.....A...F...E...G.
<b>E1</b> .....GGC.GAG.GTC.AAG.gtagtgcaagg.>10.kb.attgtctttgacag.GCT.TTT.TGA.AGG			
.....E...I...L...K(116).....			.....S...I...D...N.
<b>E2</b> 201.bp..GAG.ATC.CTG.AAA.Agtaagtag.....4.kb...tgtgactcgacagGT.ATT.GAT.AAT			
.....L...S...K...T(180).....			.....L...F...E...I.
<b>E3</b> 191.bp..CTC.AGC.AAG.ACG.gtaggtat.....9.kb..ttgtcccctttag.TTG.TTT.GAA.ATT			
.....T...L...P...Q(231).....			.....C...Q...A...A.
<b>E4</b> 152.bp..ACA.TTA.CCA.CAgtagtatg.....10.kb.gtctcccccaaaagG.TGT.CAG.GCA.GCT			
.....N...V...E...D(266).....			.....D...S...T...N.
<b>E5</b> 107.bp..AAT.GTT.GAA.GAT.Ggtaagtaaaa...350.bp.....tctagAC.TCA.ACC.AAT			
.....Q...T...C...S(378).....			.....C...Y...R...R.
<b>E6</b> 334.bp..CAA.ACA.TGC.AGgtaaggagtg.....6.kb..tttccccctagT.TGT.TAC.AGA.AGA			
.....H...L...N(415)Stop.....			.....polyA.....
<b>E7</b> 506.bp..CAT.CTG.AAC.TAA.GATCATACC...ATTGTATTATAgctgtgaag			

FIGURE 13B

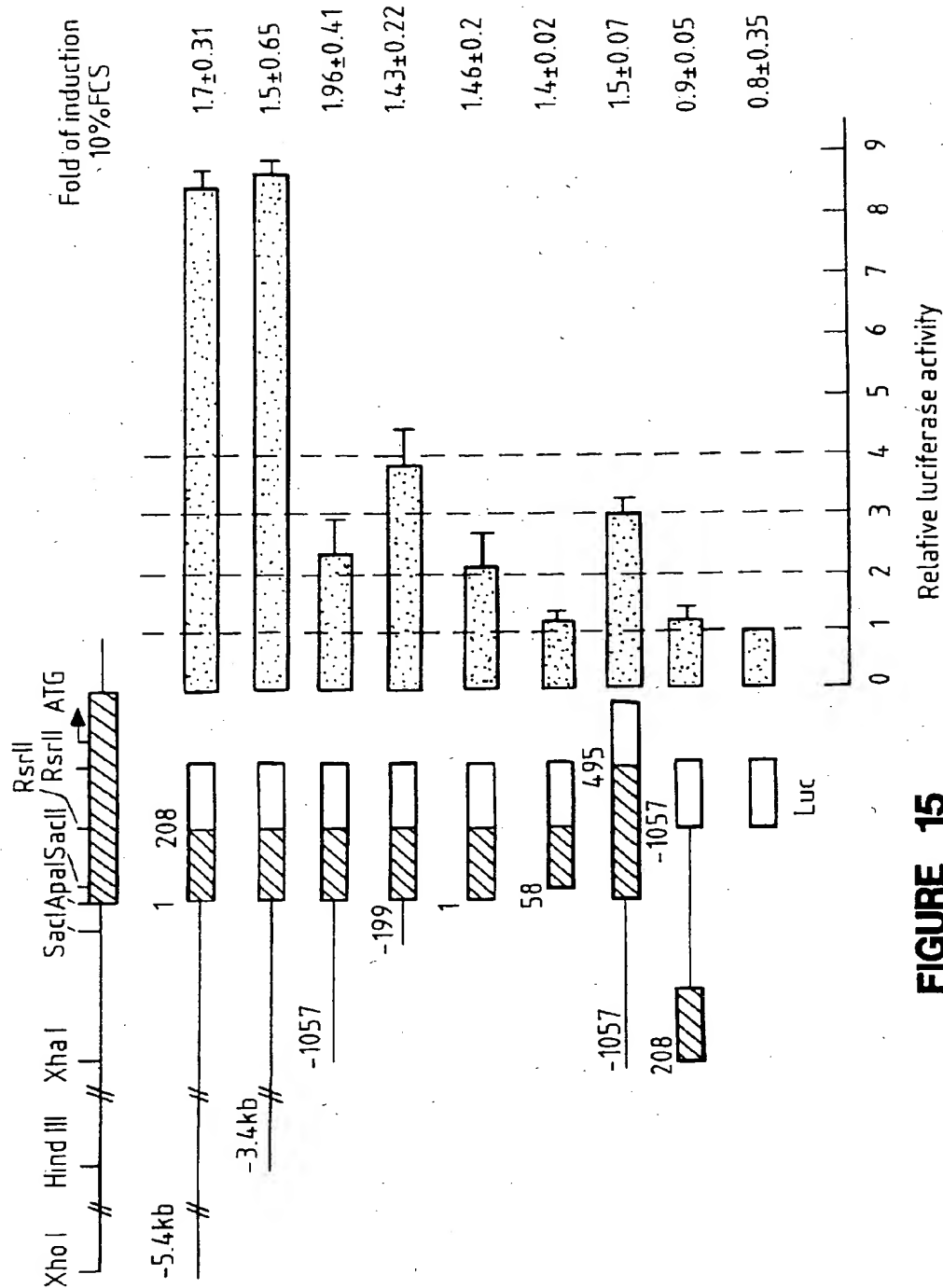
FIGURE 14A





Symbols for the conditioned media: (□) - VEGF-C R226,227S; (●) - wt VEGF-C; (○) - VEGF-CA; (▲) - VEGF-CA156S; (▼) - VEGF.

**FIGURE 14B**

**FIGURE 15**

# VASCULAR ENDOTHELIAL GROWTH FACTOR C (VEGF-C) $\Delta$ CYS<sub>156</sub> PROTEIN AND GENE, AND USES THEREOF

## FIELD OF THE INVENTION

The present invention generally relates to the field of genetic engineering and more particularly to growth factors for endothelial cells and growth factor genes.

## BACKGROUND OF THE INVENTION

Developmental growth, the remodeling and regeneration of adult tissues, as well as solid tumor growth, can only occur when accompanied by blood vessel formation. Angioblasts and hematopoietic precursor cells differentiate from the mesoderm and form the blood islands of the yolk sac and the primary vascular system of the embryo. The development of blood vessels from these early (in situ) differentiating endothelial cells is termed vasculogenesis. Major embryonic blood vessels are believed to arise via vasculogenesis, whereas the formation of the rest of the vascular tree is thought to occur as a result of vascular sprouting from pre-existing vessels, a process called angiogenesis, Risau et al., *Devel. Biol.*, 125:441-450 (1988).

Endothelial cells give rise to several types of functionally and morphologically distinct vessels. When organs differentiate and begin to perform their specific functions, the phenotypic heterogeneity of endothelial cells increases. Upon angiogenic stimulation, endothelial cells may re-enter the cell cycle, migrate, withdraw from the cell cycle and subsequently differentiate again to form new vessels that are functionally adapted to their tissue environment. Endothelial cells undergoing angiogenesis degrade the underlying basement membrane and migrate, forming capillary sprouts that project into the perivascular stroma. Ausprunk et al., *Microvasc. Res.*, 14:51-65 (1977). Angiogenesis during tissue development and regeneration depends on the tightly controlled processes of endothelial cell proliferation, migration, differentiation, and survival. Dysfunction of the endothelial cell regulatory system is a key feature of many diseases. Most significantly, tumor growth and metastasis have been shown to be angiogenesis dependent. Folkman et al., *J. Biol. Chem.*, 267:10931-10934 (1992).

Key signals regulating cell growth and differentiation are mediated by polypeptide growth factors and their transmembrane receptors, many of which are tyrosine kinases. Auto-phosphorylated peptides within the tyrosine kinase insert and carboxyl-terminal sequences of activated receptors are commonly recognized by kinase substrates involved in signal transduction for the readjustment of gene expression in responding cells. Several families of receptor tyrosine kinases have been characterized. Van der Geer et al., *Ann. Rev. Cell Biol.*, 10:251-337 (1994). The major growth factors and receptors transducing angiogenic stimuli are schematically shown in FIG. 1.

Fibroblast growth factors are also known to be involved in the regulation of angiogenesis. They have been shown to be mitogenic and chemotactic for cultured endothelial cells. Fibroblast growth factors also stimulate the production of proteases, such as collagenases and plasminogen activators, and induce tube formation by endothelial cells. Saksela et al., *Ann. Rev. Cell Biol.*, 4:93-126 (1988). There are two general classes of fibroblast growth factors, FGF-1 and FGF-2, both of which lack conventional signal peptides. Both types have an affinity for heparin and FGF-2 is bound to heparin sulfate proteoglycans in the subendothelial extracellular matrix from which it may be released after injury.

Heparin potentiates the stimulation of endothelial cell proliferation by angiogenic FGFs, both by protecting against denaturation and degradation and dimerizing the FGFs. Cultured endothelial cells express the FGF-1 receptor but no significant levels of other high-affinity fibroblast growth factor receptors.

Among other ligands for receptor tyrosine kinases, the platelet derived growth factor, PDGF-BB, has been shown to be weakly angiogenic in the chick chorioallantoic membrane. Risau et al., *Growth Factors*, 7:261-266 (1992). Transforming growth factor  $\alpha$  (TGF $\alpha$ ) is an angiogenic factor secreted by several tumor cell types and by macrophages. Hepatocyte growth factor (HGF), the ligand of the c-met proto-oncogene-encoded receptor, also is strongly angiogenic.

Recent evidence shows that there are endothelial cell specific growth factors and receptors that may be primarily responsible for the stimulation of endothelial cell growth, differentiation and certain differentiated functions. The best studied of these is vascular endothelial growth factor (VEGF), a member of the PDGF family. Vascular endothelial growth factor is a dimeric glycoprotein of disulfide-linked 23 kD subunits. Other reported effects of VEGF include the mobilization of intracellular calcium, the induction of plasminogen activator and plasminogen activator inhibitor-1 synthesis, stimulation of hexose transport in endothelial cells, and promotion of monocyte migration in vitro. Four VEGF isoforms, encoded by distinct mRNA splice variants, appear to be equally capable of stimulating mitogenesis in endothelial cells. However, each isoform has a different affinity for cell surface proteoglycans, which behave as low affinity receptors for VEGF. The 121 and 165 amino acid isoforms of VEGF (VEGF<sub>121</sub> and VEGF<sub>165</sub>) are secreted in a soluble form, whereas the isoforms of 189 and 206 amino acid residues remain cell surface-associated and have a strong affinity for heparin. VEGF was originally purified from several sources on the basis of its mitogenic activity toward endothelial cells, and also by its ability to induce microvascular permeability, hence it is also called vascular permeability factor (VPF).

The pattern of VEGF expression suggests its involvement in the development and maintenance of the normal vascular system and in tumor angiogenesis. During murine development, the entire 7.5 day post-coital (p.c.) endoderm expresses VEGF and the ventricular neuroectoderm produces VEGF at the capillary ingrowth stage. See Breier et al., *Development*, 114:521-523 (1992). On day two of quail development, the vascularized area of the yolk sac as well as the whole embryo show expression of VEGF. In addition, epithelial cells next to fenestrated endothelia in adult mice show persistent VEGF expression, suggesting a role in the maintenance of this specific endothelial phenotype and function.

Two high affinity receptors for VEGF have been characterized. These are VEGFR-1/Flt-1 (fms-like tyrosine kinase-1) and VEGFR-2/Kdr/Flk-1 (kinase insert domain containing receptor/fetal liver kinase-1). Those receptors are classified in the PDGF-receptor family, but they have seven rather than five immunoglobulin-like loops in their extracellular domain (see FIG. 1) and they possess a longer kinase insert than normally observed in this family. The expression of VEGF receptors occurs mainly in vascular endothelial cells, although some may be present on hematopoietic progenitor cells, monocytes, and melanoma cells. Only endothelial cells have been reported to proliferate in response to VEGF, and endothelial cells from different sources show different responses. Thus, the signals mediated

through VEGFR-1 and VEGFR-2 appear to be cell type specific. The VEGF-related placenta growth factor (PlGF) was recently shown to bind to VEGFR-1 with high affinity. PlGF was able to enhance the growth factor activity of VEGF, but it did not stimulate endothelial cells on its own. Naturally occurring VEGF/PlGF heterodimers were nearly as potent mitogens as VEGF homodimers for endothelial cells. Cao et al., *J. Biol. Chem.*, 271:3154-62 (1996).

The Flt4 receptor tyrosine kinase (VEGFR-3) is closely related in structure to the products of the VEGFR-1 and VEGFR-2 genes. Despite this similarity, the mature form of Flt4 differs from the VEGF receptors in that it is proteolytically cleaved in the extracellular domain into two disulfide-linked polypeptides. Pajusola et al., *Cancer Res.*, 52:5738-5743 (1992). The 4.5 and 5.8 kb Flt4 mRNAs encode polypeptides which differ in their C-termini due to the use of alternative 3' exons. Isoforms of VEGF or PlGF do not show specific binding to Flt4 or induce its autophosphorylation.

Expression of Flt4 appears to be more restricted than the expression of VEGFR-1 or VEGFR-2. The expression of Flt4 first becomes detectable by in situ hybridization in the angioblasts of head mesenchyme, the cardinal vein, and extraembryonically in the allantois of 8.5 day p.c. mouse embryos. In 12.5 day p.c. embryos, the Flt4 signal is observed in developing venous and presumptive lymphatic endothelia, but arterial endothelia appear negative. During later stages of development, Flt4 mRNA becomes restricted to developing lymphatic vessels. The lymphatic endothelia and some high endothelial venules express Flt4 mRNA in adult human tissues and increased expression occurs in lymphatic sinuses in metastatic lymph nodes and in lymphangioma. These results support the theory of the venous origin of lymphatic vessels.

Five endothelial cell specific receptor tyrosine kinases, Flt-1 (VEGFR-1), KDR/Flk-1 (VEGFR-2), Flt4 (VEGFR-3), Tie, and Tek/Tie-2 have so far been described, which possess the intrinsic tyrosine kinase activity essential for signal transduction. Targeted mutations inactivating Flt-1, Flk-1, Tie, and Tek in mouse embryos have indicated their essential and specific roles in vasculogenesis and angiogenesis at the molecular level. VEGFR-1 and VEGFR-2 bind VEGF with high affinity ( $K_d$  16 pM and 760 pM, respectively) and VEGFR-1 also binds the related placenta growth factor (PlGF;  $K_d$  about 200 pM). A ligand for Tek is reported in PCT patent publication WO 96/11269.

#### SUMMARY OF THE INVENTION

The present invention provides a ligand, designated VEGF-C, for the Flt4 receptor tyrosine kinase (VEGFR-3). Thus, the invention provides a purified and isolated polypeptide which is capable of binding to the Flt4 receptor tyrosine kinase. Preferably, an Flt4 ligand of the invention is capable of stimulating tyrosine phosphorylation of Flt4 receptor tyrosine kinase in a host cell expressing the Flt4 receptor tyrosine kinase. Preferred ligands of the invention are mammalian polypeptides. Highly preferred ligands are human polypeptides. As explained in detail below, dimers and multimers comprising polypeptides of the invention linked to each other or to other polypeptides are specifically contemplated as ligands of the invention.

In one embodiment, an Flt4 ligand polypeptide has a molecular weight of approximately 23 kD as determined by SDS-PAGE under reducing conditions. For example, the invention includes a ligand composed of one or more polypeptides of approximately 23 kD which is purifiable

from conditioned media from a PC-3 prostatic adenocarcinoma cell line, the cell line having ATCC Acc. No. CRL 1435. Amino acid sequencing of this PC-3 cell-derived ligand polypeptide revealed that the ligand polypeptide comprises an amino terminal amino acid sequence set forth in SEQ ID NO: 5. A conditioned medium comprising an Flt4 ligand is itself an aspect of the invention. The present invention also provides a new use for the PC-3 prostatic adenocarcinoma cell line which produces an Flt4 ligand. In a preferred embodiment, the ligand may be purified and isolated directly from the PC-3 cell culture medium.

In a highly preferred embodiment, the ligand polypeptide comprises a fragment of the amino acid sequence shown in SEQ ID NO: 8 which specifically binds to the human Flt4 receptor tyrosine kinase. Exemplary fragments include: a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 8 from about residue 112 to about residue 213; a polypeptide comprising an amino acid sequence from about residue 104 to about residue 227 of SEQ ID NO: 8; and a polypeptide comprising an amino acid sequence from about residue 112 to about residue 227 of SEQ ID NO: 8. Other exemplary fragments include polypeptides comprising amino acid sequences of SEQ ID NO: 8 that span, approximately, the following residues: 31-213, 31-227, 32-227, 103-217, 103-225, 104-213, 113-213, 103-227, 113-227, 131-211, 161-211, 103-225, 227-419, 228-419, 31-419, and 1-419, as described in greater detail below.

The present invention also provides one or more polypeptide precursors of an Flt4 ligand, wherein one such precursor (designated "prepro-VEGF-C") comprises the complete amino acid sequence (amino acid residues 1 to 419) shown in SEQ ID NO: 8. Thus, the invention includes a purified and isolated polypeptide having the amino acid sequence of residues 1 to 419 shown in SEQ ID NO: 8. Ligand precursors according to the invention, when expressed in an appropriate host cell, produce, via cleavage, a polypeptide which binds specifically to the Flt4 receptor tyrosine kinase. A putative 102 amino acid leader (prepro) peptide has been identified in the amino acid sequence shown in SEQ ID NO: 8. Thus, in a related aspect, the invention includes a purified and isolated polypeptide having the amino acid sequence of residues 103-419 shown in SEQ ID NO: 8.

In one embodiment, an expressed Flt4 ligand polypeptide precursor is proteolytically cleaved upon expression to produce an approximately 23 kD Flt4 ligand polypeptide. Thus, an Flt4 ligand polypeptide is provided which is the cleavage product of the precursor polypeptide shown in SEQ ID NO: 8 and which has a molecular weight of approximately 23 kD under reducing conditions.

Putative VEGF-C precursors/processing products consisting of polypeptides with molecular weights of about 29 and 32 kD also are considered aspects of the invention.

In another embodiment, an expressed Flt4 ligand polypeptide precursor is proteolytically cleaved upon expression to produce an approximately 21 kD VEGF-C polypeptide. Sequence analysis has indicated that an observed 21 kD form has an amino terminus approximately 9 amino acids downstream from the amino terminus of the 23 kD form, suggesting that alternative cleavage sites exist.

From the foregoing, it will be apparent that an aspect of the invention includes a fragment of the purified and isolated polypeptide having the amino acid sequence of residues 1 to 419 shown in SEQ ID NO: 8, the fragment being capable of specifically binding to Flt4 receptor tyrosine kinase. Preferred embodiments include fragments having an apparent molecular weight of approximately 21/23 kD and 29/32 kD



as assessed by SDS-PAGE under reducing conditions. More generally, the invention includes a purified and isolated polypeptide according to claim 1 that is a VEGF-C of vertebrate origin, wherein the VEGF-C has a molecular weight of about 21–23 kD, as assessed by SDS-PAGE under reducing conditions, and wherein the VEGF-C is capable of binding to Flt4 receptor tyrosine kinase (VEGFR-3).

Evidence suggests that the amino acids essential for retaining Flt4 ligand activity are contained within approximately amino acids 103/112–226/227 of SEQ ID NO: 8, and that a carboxy-terminal proteolytic cleavage to produce a mature, naturally-occurring Flt4 ligand occurs at the approximate position of amino acids 226–227 of SEQ ID NO: 8. Accordingly, a preferred Flt4 ligand comprises approximately amino acids 103–227 of SEQ ID NO: 8.

VEGF-C mutational analysis described herein indicates that a naturally occurring VEGF-C polypeptide spanning amino acids 103–227 of SEQ ID NO: 8, produced by a natural processing cleavage that defines the C-terminus, exists and is biologically active as an Flt4 ligand. A polypeptide fragment consisting of residues 104–213 of SEQ ID NO: 8 has been shown to retain VEGF-C biological activity. Additional mutational analyses indicate that a polypeptide spanning only amino acids 113–213 of SEQ ID NO: 8 retains Flt4 ligand activity. Accordingly, preferred polypeptides comprise sequences spanning, approximately, amino acid residues 103–227, 104–213, or 113–213, of SEQ ID NO: 8.

Moreover, sequence comparisons of members of the VEGF family of polypeptides provide an indication that still smaller fragments will retain biological activity, and such smaller fragments are intended as aspects of the invention. In particular, eight highly conserved cysteine residues of the VEGF family of polypeptides define a region from residue 131 to residue 211 of SEQ ID NO: 8 (see FIGS. 10 & 32); therefore, a polypeptide spanning from about residue 131 to about residue 211 is expected to retain VEGF-C biological activity. In fact, a polypeptide comprising approximately residues 161–211, which retains an evolutionarily-conserved RCXXCC motif, is postulated to retain VEGF-C activity, and therefore is intended as an aspect of the invention.

In addition to binding Flt4, VEGF-C polypeptides are shown herein to bind and activate kdr/flk-1 receptor tyrosine kinase (VEGFR-2). Thus, the invention includes a purified and isolated polypeptide that is capable of binding to at least one of kdr receptor tyrosine kinase (VEGFR-2) and Flt4 receptor tyrosine kinase (VEGFR-3), the polypeptide comprising a portion of the amino acid sequence in SEQ ID NO: 8 effective to permit such binding. In one preferred embodiment, the portion of the amino acid sequence in SEQ ID NO: 8 is a continuous portion having as its amino terminal residue an amino acid between residues 102 and 161 of SEQ ID NO: 8 and having as its carboxy terminal residue an amino acid between residues 210 and 228 of SEQ ID NO: 8. In a highly preferred embodiment, the portion has, as its amino terminal residue, an amino acid between residues 102 and 131 of SEQ ID NO: 8. In a very highly preferred embodiment, the portion of the amino acid sequence in SEQ ID NO: 8 is a continuous portion having as its amino terminal residue an amino acid between residues 102 and 114 of SEQ ID NO: 8 and having as its carboxy terminal residue an amino acid between residues 212 and 228 of SEQ ID NO: 8. Polypeptides of the invention which bind to and activate a receptor (e.g., VEGFR-2 or VEGFR-3) are useful for stimulating VEGF-C biological activities that are mediated through the receptor. Polypeptides of the invention which bind to but do not activate a receptor are useful for inhibiting VEGF-C activities mediated through that receptor.

The definition of polypeptides of the invention is intended to include within its scope variants thereof. The polypeptide variants contemplated include purified and isolated polypeptides having amino acid sequences that differ from the exact amino acid sequences of such polypeptides (e.g., VEGF-C, VEGF-C precursors and VEGF-C fragments) by conservative substitutions, as recognized by those of skill in the art, that are compatible with the retention of at least one VEGF-C biological activity or VEGF-C-inhibitory activity of the polypeptide. The term "variants," when used to refer to polypeptides, also is intended to include polypeptides having amino acid additions, including but not limited to additions of a methionine and/or leader sequence to promote translation and/or secretion; additions of peptide sequences to facilitate purification (e.g., polyhistidine sequences and/or epitopes for antibody purification); and additions of polypeptide-encoding sequences to produce fusion proteins with VEGF-C. The term "variants" also is intended to include polypeptides having amino acid deletions at the amino terminus, the carboxy terminus, or internally of amino acids that are non-conserved amongst the human, mouse, and quail VEGF-C sequences taught herein, and that are compatible with the retention of the VEGF-C or VEGF-C-inhibitory activity of the polypeptide to which the deletions have been made.

The term "variant" also is intended to include polypeptides having modifications to one or more amino acid residues that are compatible with retaining VEGF-C or VEGF-C inhibitory activity of the polypeptide. Such modifications include glycosylations (identical or different to glycosylations of native VEGF-C); and the addition of other substituents (e.g., labels, compounds to increase serum half-life (e.g., polyethylene glycol), and the like.

Additional polypeptides of the invention include certain fragments that have been observed to result from the processing of prepro-VEGF-C into mature VEGF-C. For example, the invention includes a purified and isolated polypeptide having a molecular weight of about 29 kD as assessed by SDS-PAGE under reducing conditions and having an amino acid sequence consisting essentially of a portion of SEQ ID NO: 8 having residue 228 of SEQ ID NO: 8 as its amino terminal amino acid residue; and a purified and isolated polypeptide having a molecular weight of about 15 kD as assessed by SDS-PAGE under reducing conditions and having an amino acid sequence consisting essentially of a portion of SEQ ID NO: 8 having residue 32 of SEQ ID NO: 8 as its amino terminal amino acid residue. Such polypeptides are expected to modulate VEGF-C biological activity through their interactions with VEGF-C receptors and/or interactions with biologically active VEGF-C.

Some of the conserved cysteine residues in VEGF-C participate in interchain disulfide bonding to make homo- and heterodimers of the various naturally occurring VEGF-C polypeptides. Beyond the preceding considerations, evidence exists that VEGF-C polypeptides lacking interchain disulfide bonds retain VEGF-C biological activity. Consequently, the materials and methods of the invention include all VEGF-C fragments that retain at least one biological activity of VEGF-C, regardless of the presence or absence of interchain disulfide bonds. The invention also includes multimers (including dimers) comprising such fragments linked to each other or to other polypeptides. Fragment linkage may be by way of covalent bonding (e.g., disulfide bonding) or non-covalent bonding of polypeptide chains (e.g., hydrogen bonding, bonding due to stable or induced dipole-dipole interactions, bonding due to hydrophobic or hydrophilic interactions, combinations of these

bonding mechanisms, and the like). Thus, the invention includes a purified and isolated polypeptide multimer, wherein at least one monomer thereof is a polypeptide that is capable of binding to VEGFR-2 and/or VEGFR-3, the polypeptide comprising a portion of the amino acid sequence in SEQ ID NO: 8 effective to permit such binding, and wherein the multimer itself is capable of binding to VEGFR-2 and/or VEGFR-3. In a preferred embodiment, the multimer has at least one VEGF-C biological activity as taught herein.

In one embodiment, at least one monomer of the multimer is a polypeptide from another member of the PDGF/VEGF family of proteins, e.g., a vascular endothelial growth factor (VEGF) polypeptide, a vascular endothelial growth factor B (VEGF-B) polypeptide, a platelet derived growth factor A (PDGF-A) polypeptide, a platelet derived growth factor B (PDGF-B) polypeptide, a c-fos induced growth factor (FIGF) polypeptide, or a placental growth factor (PIGF) polypeptide.

In a highly preferred embodiment, the multimer of the invention is a dimer of two monomer polypeptides. For example, the invention includes a dimer wherein each monomer thereof is capable of binding to at least one of VEGFR-2 and VEGFR-3 and has an amino acid sequence comprising a portion of SEQ ID NO: 8 effective to permit such binding. Dimers having covalent attachments and dimers wherein the two monomers are free of covalent attachments to each other are contemplated.

In yet another aspect, the invention includes analogs of the polypeptides of the invention. The term "analog" refers to polypeptides having alterations involving one or more amino acid insertions, internal amino acid deletions, and/or non-conservative amino acid substitutions (replacements). The definition of analog is intended to include within its scope variants of analog polypeptides embodying such alterations. The term "mutant," when used with respect to polypeptides herein, is intended to refer generically to VEGF-C variants, VEGF-C analogs, and variants of VEGF-C analogs.

For example, in one embodiment, the invention includes a polypeptide analog of a VEGF-C of vertebrate origin that is capable of binding to VEGFR-3 (e.g., an analog of a vertebrate VEGF-C of about 21-23 kD as assessed by SDS-PAGE under reducing conditions), wherein an evolutionarily conserved cysteine residue in the VEGF-C has been deleted or replaced, and wherein the analog is capable of binding to VEGFR-3 and has reduced VEGFR-2 binding affinity relative to the wildtype VEGF-C. For analogs according to this embodiment of the invention, the determination that a residue is "evolutionarily conserved" is made solely by reference to the alignment of human, mouse, and quail VEGF-C sequences provided herein and aligned to show similarity in FIG. 5. The presence of the same residue in all three sequences indicates that the residue is evolutionarily conserved, notwithstanding the fact that VEGF-C from other species may lack the residue. In a preferred embodiment, the conserved cysteine residue corresponds to the cysteine at position 156 of SEQ ID NO: 8. "Correspondence to the cysteine at position 156" is readily determined from an analysis of the vertebrate VEGF-C sequence of interest, since the cysteine at position 156 of SEQ ID NO: 8 (human VEGF-C) falls within an evolutionarily conserved portion of VEGF-C (see FIG. 5, comparing human, mouse, and quail VEGF-C polypeptides). Alignment of human VEGF-C allelic variants, other mammalian VEGF-C polypeptides, and the like with the three VEGF-C forms in FIG. 5 will identify that cysteine which corresponds to the

cysteine at position 156 of SEQ ID NO: 8, even if the allelic variant has greater or fewer than exactly 155 residues preceding the cysteine of interest.

In another embodiment, the invention includes a purified polypeptide that is an analog of human VEGF-C and that is capable of binding to at least one of fit-1 receptor tyrosine kinase (VEGFR-1), kdr receptor tyrosine kinase (VEGFR-2), and Flt4 receptor tyrosine kinase (VEGFR-3).

Specifically contemplated is an analog of human VEGF-C that binds VEGFR-3 but has reduced VEGFR-2 binding affinity, as compared to the VEGFR-2 binding affinity of a wildtype human VEGF-C (e.g., as compared to the VEGFR-2 binding affinity of a human VEGF-C having an amino acid sequence consisting essentially of amino acids 103-227 of SEQ ID NO: 8). One such family of human VEGF-C analogs are VEGF-C  $\Delta_{156}$  polypeptides. By "VEGF-C  $\Delta_{156}$  polypeptide" is meant an analog wherein the cysteine at position 156 of SEQ ID NO: 8 has been deleted or replaced by another amino acid. A VEGF-C  $\Delta_{156}$  polypeptide analog can be made from any VEGF-C polypeptide of the invention that comprises all of SEQ ID NO: 8 or a portion thereof that includes position 156 of SEQ ID NO: 8. Preferably, the VEGF-C  $\Delta_{156}$  polypeptide analog comprises a portion of SEQ ID NO: 8 effective to permit binding to VEGFR-3.

For example, the invention includes a VEGF-C  $\Delta_{156}$  polypeptide that binds VEGFR-3, has reduced VEGFR-2 binding affinity, and has an amino acid sequence which includes amino acids 131 to 211 of SEQ ID NO: 8, wherein the cysteine residue at position 156 of SEQ ID NO: 8 has been deleted or replaced. In a preferred embodiment, the VEGF-C  $\Delta_{156}$  polypeptide comprises a continuous portion of SEQ ID NO: 8, the portion having as its amino terminal residue an amino acid between residues 102 and 114 of SEQ ID NO: 8, and having as its carboxy terminal residue an amino acid between residues 212 and 228 of SEQ ID NO: 8, wherein the cysteine residue at position 156 of SEQ ID NO: 8 has been deleted or replaced. In an embodiment exemplified herein, the cysteine residue at position 156 of SEQ ID NO: 8 has been replaced by a serine residue.

A second family of human VEGF-C analogs that bind VEGFR-3 but have reduced VEGFR-2 binding affinity are VEGF-C  $\Delta R_{226}\Delta R_{227}$  polypeptides. By "VEGF-C  $\Delta R_{226}\Delta R_{227}$  polypeptide" is meant an analog wherein the arginine residues at positions 226 and 227 of SEQ ID NO: 8 have been deleted or replaced by other amino acids, for the purpose of eliminating a proteolytic processing site of the carboxy terminal pro-peptide of VEGF-C. Preferably, the VEGF-C  $\Delta R_{226}\Delta R_{227}$  polypeptide comprises a portion of SEQ ID NO: 8 effective to permit binding of VEGFR-3. For example, the invention includes a VEGF-C  $\Delta R_{226}\Delta R_{227}$  polypeptide having an amino acid sequence comprising amino acids 112-419 of SEQ ID NO: 8, wherein the arginine residues at positions 226 and 227 of SEQ ID NO: 8 have been deleted or replaced. Specifically exemplified herein is a VEGF-C  $\Delta R_{226}\Delta R_{227}$  polypeptide wherein the arginine residues at positions 226 and 227 of SEQ ID NO: 8 have been replaced by serine residues.

Another family of VEGF-C analogs of the invention are human VEGF-C<sup>basic</sup> polypeptides. By "VEGF-C<sup>basic</sup> polypeptide" is meant a VEGF-C analog wherein at least one amino acid having a basic side chain has been introduced into the VEGF-C coding sequence, to emulate one or more basic residues in VEGF (e.g., residues Arg<sub>108</sub>, Lys<sub>110</sub>, and His<sub>112</sub> in the VEGF<sub>165</sub> precursor shown in FIG. 2.) that have been implicated in VEGF receptor binding. Preferably,

two or three basic residues are introduced into VEGF-C. Based on the VEGF-VEGF-C polypeptide alignment provided herein, positions 187, 189, and 191 of SEQ ID NO: 8 are preferred positions to introduce basic residues. For example, the invention includes a VEGF-C<sup>basic</sup> polypeptide that is capable of binding to at least one of VEGFR-1, VEGFR-2, and VEGFR-3, and that has an amino acid sequence comprising residues 131 to 211 of SEQ ID NO: 8, wherein the glutamic acid residue at position 187, the threonine residue at position 189, and the proline residue at position 191 of SEQ ID NO: 8 have been replaced by an arginine residue, a lysine residue, and a histidine residue, respectively.

In yet another aspect of the invention, VEGF-C structural information is employed to create useful analogs of VEGF. For example, mature VEGF-C contains an unpaired cysteine (position 137 of SEQ ID NO: 8) and is able to form non-covalently bonded polypeptide dimers. In one embodiment, a VEGF analog is created wherein this unpaired cysteine residue from mature VEGF-C is introduced at an analogous position of VEGF (e.g., introduced in place of Leu<sub>58</sub> of the human VEGF165 precursor (FIG. 2, Genbank Acc. No. M32977). Such VEGF analogs are termed VEGF<sup>Cys</sup> polypeptides. Thus, the invention includes a human VEGF analog wherein a cysteine residue is introduced in the VEGF amino acid sequence at a position selected from residues 53 to 63 of the human VEGF165 precursor having the amino acid sequence set forth in SEQ ID NO: 56. At least four naturally occurring VEGF isoforms have been described, and VEGF<sup>Cys</sup> polypeptide analogs of each isoform are contemplated. Most preferably, the cysteine is introduced at a position in a VEGF isoform which corresponds to position 58 of the VEGF165 precursor having the amino acid sequence set forth in SEQ ID NO: 56.

The present invention also provides purified and isolated polynucleotides (i.e., nucleic acids) encoding all of the polypeptides of the invention, including but not limited to cDNAs and genomic DNAs encoding VEGF-C precursors, VEGF-C, and biologically active fragments thereof, and DNAs encoding VEGF-C variants and VEGF-C analogs. A preferred nucleic acid of the invention comprises a DNA encoding amino acid residues 1 to 419 of SEQ ID NO: 8 or one of the aforementioned fragments or analogs thereof. Due to the degeneracy of the genetic code, numerous such coding sequences are possible, each having in common the coding of the amino acid sequence shown in SEQ ID NO: 8 or the fragment or analog thereof. Distinct polynucleotides encoding any polypeptide of the invention by virtue of the degeneracy of the genetic code are within the scope of the invention.

A preferred polynucleotide according to the invention comprises the human VEGF-C cDNA sequence set forth in SEQ ID NO: 7 from nucleotide 352 to 1611. Other polynucleotides according to the invention encode a VEGF-C polypeptide from, e.g., mammals other than humans, birds (e.g., avian quails), and others. Still other polynucleotides of the invention comprise a coding sequence for a VEGF-C fragment, and allelic variants of those DNAs encoding part or all of VEGF-C. Still other polynucleotides of the invention comprise a coding sequence for a VEGF-C variant or a VEGF-C analog.

The invention further comprises polynucleotides that hybridize to the aforementioned polynucleotides under standard stringent hybridization conditions. Exemplary stringent hybridization conditions are as follows: hybridization at 42° C. in 50% formamide, 5×SSC, 20 mM Na<sub>2</sub>PO<sub>4</sub>, pH 6.8 and washing in 0.2×SSC at 55° C. It is understood by those of

skill in the art that variation in these conditions occurs based on the length and GC nucleotide content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining appropriate hybridization conditions. See Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Second ed., Cold Spring Harbor Laboratory Press, 1989) §§ 9.47-9.51. These polynucleotides, capable of hybridizing to polynucleotides encoding VEGF-C, VEGF-C fragments, or VEGF-C analogs, are useful as nucleic acid probes for identifying, purifying and isolating polynucleotides encoding other (non-human) mammalian forms of VEGF-C and human VEGF-C allelic variants. Additionally, these polynucleotides are useful in screening methods of the invention, as described below.

Preferred nucleic acids useful as probes of the invention comprise nucleic acid sequences of at least about 16 continuous nucleotides of SEQ ID NO: 7. More preferably, these nucleic acid probes would have at least about 20 continuous nucleotides found in SEQ ID NO: 7. In using these nucleic acids as probes, it is preferred that the nucleic acids specifically hybridize to a portion of the sequence set forth in SEQ ID NO: 7. Specific hybridization is herein defined as hybridization under standard stringent hybridization conditions. To identify and isolate other mammalian VEGF-C genes specifically, nucleic acid probes preferably are selected such that they fail to hybridize to genes related to VEGF-C (e.g., fail to hybridize to human VEGF or to human VEGF-B genes).

Thus, the invention comprehends polynucleotides comprising at least about 16 nucleotides wherein the polynucleotides are capable of specifically hybridizing to a gene encoding VEGF-C, e.g., a human gene. The specificity of hybridization ensures that a polynucleotide of the invention is able to hybridize to a nucleic acid encoding a VEGF-C under hybridization conditions that do not support hybridization of the polynucleotide to nucleic acids encoding, e.g., VEGF or VEGF-B. In one embodiment, polynucleotides of at least about 16 nucleotides, and preferably at least about 20 nucleotides, are selected as continuous nucleotide sequences found in SEQ ID NO: 7 or the complement of the nucleotide sequence set forth in SEQ ID NO: 7.

In another embodiment, the invention includes polynucleotides having at least 90 percent (preferably at least 95 percent, and more preferably at least 97, 98, or 99 percent) nucleotide sequence identity with a nucleotide sequence encoding a polypeptide of the invention. In a highly preferred embodiment, the polynucleotides have at least 95 percent sequence identity with a nucleotide sequence encoding a human VEGF-C precursor (such as the VEGF-C precursor in SEQ ID NO: 8 and allelic variants thereof), human VEGF-C, or biologically active VEGF-C fragments.

Additional aspects of the invention include vectors which comprise nucleic acids of the invention; and host cells transformed or transfected with nucleic acids or vectors of the invention. Preferred vectors of the invention are expression vectors wherein nucleic acids of the invention are operatively connected to appropriate promoters and other control sequences that regulate transcription and/or subsequent translation, such that appropriate prokaryotic or eukaryotic host cells transformed or transfected with the vectors are capable of expressing the polypeptide encoded thereby (e.g., the VEGF-C, VEGF-C fragment, VEGF-C variant, or VEGF-C analog encoded thereby). A preferred vector of the invention is plasmid pFLT4-L, having ATCC accession no. 97231. Such vectors and host cells are useful for recombinantly producing polypeptides of the invention, including VEGF-C, and fragments, variants, and analogs thereof.

In a related aspect of the invention, host cells such as procaryotic and eukaryotic cells, especially unicellular host cells, are modified to express polypeptides of the invention. Host cells may be stably transformed or transfected with isolated DNAs of the invention in a manner allowing expression of polypeptides of the invention therein. Thus, the invention further includes a method of making polypeptides of the invention. In a preferred method, a nucleic acid or vector of the invention is expressed in a host cell, and a polypeptide of the invention is purified from the host cell or the host cell's growth medium.

Similarly, the invention includes a method of making a polypeptide capable of specifically binding to VEGFR-1, VEGFR-2 and/or VEGFR-3, comprising the steps of: (a) transforming or transfecting a host cell with a nucleic acid of the invention; (b) cultivating the host cell to express the nucleic acid; and (c) purifying a polypeptide capable of specifically binding to VEGFR-1, VEGFR-2, and/or VEGFR-3 from the host cell or from the host cell's growth media. The invention also includes purified and isolated polypeptides produced by methods of the invention. In one preferred embodiment, the invention includes a human VEGF-C polypeptide or biologically active fragment, variant, or analog thereof that is substantially free of other human polypeptides.

Alternatively, host cells may be modified by activating an endogenous VEGF-C gene that is not normally expressed in the host cells or that is expressed at a lower rate than is desired. Such host cells are modified (e.g., by homologous recombination) to express the VEGF-C by replacing, in whole or in part, the naturally-occurring VEGF-C promoter with part or all of a heterologous promoter so that the host cells express VEGF-C. In such host cells, the heterologous promoter DNA is operatively linked to the VEGF-C coding sequences, i.e., controls transcription of the VEGF-C coding sequences. See, for example, PCT International Publication No. WO 94/12650; PCT International Publication No. WO 92/20808; and PCT International Publication No. WO 91/09955. The invention also contemplates that, in addition to heterologous promoter DNA, amplifiable marker DNA (e.g., *ada*, *dhfr*, and the multifunctional CAD gene which encodes carbamyl phosphate synthase, aspartate transcarbamylase, and dihydro-orotase) and/or intron DNA may be recombined along with the heterologous promoter DNA into the host cells. If linked to the VEGF-C coding sequences, amplification of the marker DNA by standard selection methods results in co-amplification of the VEGF-C coding sequences in such host cells. Thus, the invention includes, for example, a cell comprising a nucleic acid having a sequence encoding human VEGF-C and further comprising a non-VEGF-C promoter sequence (i.e., a heterologous promoter sequence) or other non-VEGF-C control sequence that increases RNA transcription in the cell of the sequence encoding human VEGF-C.

The DNA sequence information provided by the present invention also makes possible the development, by homologous recombination or "knockout" strategies [see, Capecchi, *Science*, 244: 1288-1292 (1989)], of rodents that fail to express functional VEGF-C or that express a VEGF-C fragment, variant, or analog. Such rodents are useful as models for studying the activities of VEGF-C and VEGF-C modulators in vivo.

In another aspect, the invention includes an antibody which is specifically reactive with one or more polypeptides of the invention, and/or is reactive with polypeptide multimers of the invention. Antibodies, both monoclonal and polyclonal, may be made against a polypeptide of the

invention according to standard techniques in the art. See, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1988)). Standard protein manipulation techniques and recombinant techniques also may be employed to generate humanized antibodies and antigen-binding antibody fragments and other chimeric antibody polypeptides, all of which are considered antibodies of the invention. The invention further includes hybridoma cells that produce antibodies of the invention or other cell types that have been genetically engineered to express antibody polypeptides of the invention. Antibodies of the invention may be used in diagnostic applications to monitor angiogenesis, vascularization, lymphatic vessels and their disease states, wound healing, or certain tumor cells, hematopoietic, or leukemia cells. The antibodies also may be used to block the ligand from activating its receptors; to purify polypeptides of the invention; and to assay fluids for the presence of polypeptides of the invention. The invention further includes immunological assays (including radio-immuno assays, enzyme linked immunosorbent assays, sandwich assays and the like) which employ antibodies of the invention.

Ligands according to the invention may be labeled with a detectable label and used to identify their corresponding receptors in situ. Labeled Flt4 ligand and anti-Flt4 ligand antibodies may be used as imaging agents in the detection of lymphatic vessels, high endothelial venules and their disease states, and Flt4 receptors expressed in histochemical tissue sections. The ligand or antibody may be covalently or non-covalently coupled to a suitable supermagnetic, paramagnetic, electron dense, echogenic, or radioactive agent for imaging. Other, non-radioactive labels, such as biotin and avidin, may also be used.

A related aspect of the invention is a method for the detection of specific cells, e.g., endothelial cells. These cells may be found in vivo, or in ex vivo biological tissue samples. The method of detection comprises the steps of contacting a biological tissue comprising, e.g., endothelial cells, with a polypeptide according to the invention which is capable of binding to VEGFR-2 and/or VEGFR-3; under conditions wherein the polypeptide binds to the cells, optionally washing the biological tissue, and detecting the polypeptide bound to the cells in the biological tissue, thereby detecting the cells. It will be apparent that certain polypeptides of the invention are useful for detecting and/or imaging cells that express both VEGFR-2 and VEGFR-3, whereas other polypeptides (e.g., VEGF-C  $\Delta C_{156}$  polypeptides) are useful for imaging specifically those cells which express VEGFR-3.

The many biological activities described herein for VEGF-C (including but not limited to affecting growth and migration of vascular endothelial cells; promoting growth of lymphatic endothelial cells and lymphatic vessels; increasing vascular permeability; and affecting myelopoiesis (e.g., growth of neutrophilic granulocytes)) support numerous diagnostic and in vitro and in vivo clinical utilities for polypeptides and antibodies of the invention, for modulating (stimulating or inhibiting) these biological activities. Generally, VEGF-C and precursor, fragment, variant, and analog polypeptides that retain one or more VEGF-C biological activities are useful agonists for stimulating the desired biological activity; whereas precursor, fragment, variant, and analog polypeptides that are capable of binding to VEGFR-2 and/or VEGFR-3 (either alone or as a homo- or hetero-dimer with other polypeptides) without stimulating receptor-mediated VEGF-C activity (i.e., without activating the receptor) are useful as antagonists (inhibitors) of

VEGF-C. Similarly, antibodies of the invention that bind biologically active VEGF-C forms and thereby interfere with VEGF-C-receptor interactions are useful as inhibitors of VEGF-C. Antisense oligonucleotides comprising a portion of the VEGF-C coding sequence and/or its complement also are contemplated as inhibitors of the invention. Both biologically active polypeptides and inhibitor polypeptides of the invention have utilities in various imaging applications.

For example, the biological effects of VEGF-C on vascular endothelial cells indicate *in vivo* uses for polypeptides of the invention for stimulating angiogenesis (e.g., during wound healing, in tissue transplantation, in eye diseases, in the formation of collateral vessels around arterial stenoses and into injured tissues after infarction) and for inhibiting angiogenesis (e.g., to inhibit tumor growth and/or metastatic cancer). The biological effects on vascular endothelial cells indicate *in vitro* uses for biologically active forms of VEGF-C to promote the growth of cultured vascular endothelial cells.

The biological effects of VEGF-C on lymphatic endothelial cells indicate *in vivo* uses for polypeptides of the invention for stimulating lymphangiogenesis (e.g., to promote re-growth or permeability of lymphatic vessels in, for example, organ transplant patients; to mitigate the loss of axillary lymphatic vessels following surgical interventions in the treatment of cancer (e.g., breast cancer); to treat aplasia of the lymphatic vessels or lymphatic obstructions) and for inhibiting it (e.g., to treat lymphangiomas). Additional *in vivo* uses for polypeptides of the invention include the treatment or prevention of inflammation, edema, elephantiasis, and Milroy's disease. The biological effects on lymphatic endothelial cells indicate *in vitro* uses for biologically active forms of VEGF-C to promote the growth of cultured lymphatic endothelial cells.

Thus, the invention includes a method of modulating (stimulating/increasing or inhibiting/decreasing) the growth of vertebrate endothelial cells comprising contacting such endothelial cells with a polypeptide or antibody (or antigen-binding portion thereof) of the invention, in an amount effective to modulate the growth of the endothelial cells. Mammalian endothelial cells are preferred. Human endothelial cells are highly preferred. In one embodiment, the endothelial cells are lymphatic endothelial cells. In another embodiment, the cells are vascular endothelial cells. The method may be an *in vitro* method (e.g., for cultured endothelial cells) or an *in vivo* method. For *in vivo* methods, it is highly preferable to administer a pharmaceutical composition (comprising the polypeptide formulated in a pharmaceutically acceptable diluent, adjuvant, excipient, carrier, or the like) to the subject, in an amount effective to modulate the growth of lymphatic endothelial cells *in vivo*.

In one preferred embodiment, the endothelial cells are lymphatic endothelial cells, and the polypeptide is one that has reduced effect on the permeability of mammalian blood vessels compared to a wildtype VEGF-C polypeptide (e.g., compared with VEGF-C having an amino acid sequence set forth in SEQ ID NO: 8 from residue 103 to residue 227). VEGF-C  $\Delta C_{156}$  polypeptides are contemplated for use in this embodiment.

In modulating the growth of endothelial cells *in vivo*, the invention contemplates the modulation of endothelial cell-related disorders. Endothelial cell disorders contemplated by the invention include, but are not limited to, physical loss of lymphatic vessels (e.g., surgical removal of axillary lymph tissue), lymphatic vessel occlusion (e.g., elephantiasis), and

lymphangiomas. In a preferred embodiment, the subject, and endothelial cells, are human. The endothelial cells may be provided *in vitro* or *in vivo*, and they may be contained in a tissue graft. An effective amount of a polypeptide is defined herein as that amount of polypeptide empirically determined to be necessary to achieve a reproducible change in cell growth rate (as determined by microscopic or macroscopic visualization and estimation of cell doubling time, or nucleic acid synthesis assays), as would be understood by one of ordinary skill in the art.

Polypeptides of the invention may be used to stimulate lymphocyte production and maturation, and to promote or inhibit trafficking of leukocytes between tissues and lymphatic vessels or to affect migration in and out of the thymus.

The biological effects of VEGF-C on myelopoiesis indicate *in vivo* and *in vitro* uses for polypeptides of the invention for stimulating myelopoiesis (especially growth of neutrophilic granulocytes) or inhibiting it. Thus, the invention includes a method for modulating myelopoiesis in a mammalian subject comprising administering to a mammalian subject in need of modulation of myelopoiesis an amount of a polypeptide or antibody (or antigen-binding portion thereof) of the invention that is effective to modulate myelopoiesis. In one embodiment, a mammalian subject suffering from granulocytopenia is selected, and the method comprises administering to the subject an amount of a polypeptide effective to stimulate myelopoiesis. In particular, a polypeptide of the invention is administered in an amount effective to increase the neutrophil count in blood of the subject. Preferred subjects are human subjects. An effective amount of a polypeptide is an amount of polypeptide empirically determined to be necessary to achieve a reproducible change in the production of neutrophilic granulocytes (as determined by microscopic or macroscopic visualization and estimation of cell doubling time, or nucleic acid synthesis assays), as would be understood by one of ordinary skill in the art.

In a related embodiment, the invention includes a method of increasing the number of neutrophils in the blood of a mammalian subject comprising the step of expressing in a cell in a subject in need of an increased number of blood neutrophils a DNA encoding a VEGF-C protein, the DNA operatively linked to a non-VEGF-C promoter or other non-VEGF-C control sequence that promotes expression of the DNA in the cell.

Similarly, the invention includes a method of modulating the growth of neutrophilic granulocytes *in vitro* or *in vivo* comprising the step of contacting mammalian stem cells with a polypeptide or antibody of the invention in an amount effective to modulate the growth of mammalian endothelial cells.

For methods which involve the *in vivo* administration of polypeptides or antibodies of the invention, it is contemplated that the polypeptides or antibodies will be administered in any suitable manner using an appropriate pharmaceutically-acceptable vehicle, e.g., a pharmaceutically-acceptable diluent, adjuvant, excipient or carrier. Thus, the invention further includes compositions, e.g., pharmaceutical compositions, comprising one or more polypeptides or antibodies of the invention. By pharmaceutical composition is meant a composition that may be administered to a mammalian host, e.g., orally, topically, parenterally (including subcutaneous injections, intravenous, intramuscular, intracisternal injection or infusion techniques), by inhalation spray, or rectally, in unit dosage formulations containing conventional non-toxic

carriers, diluents (e.g., calcium carbonate, sodium carbonate, lactose, calcium phosphate, sodium phosphate, kaolin, water), adjuvants, vehicles, and the like, including but not limited to flavoring agents, preserving agents; granulating and disintegrating agents; binding agents; time delay materials; oils; suspending agents; dispersing or wetting agents; anti-oxidants; emulsifiers, etc.

The invention further provides a method of using a polypeptide of the invention for the manufacture of a medicament for use in any of the foregoing methods. Similarly, the invention further provides a method of using a polypeptide of the invention for the manufacture of a medicament for the treatment of any of the foregoing indicated conditions and disease states. Such methods optionally involve the use of additional biologically active ingredients (e.g., VEGF, PlGF, G-CSF, etc.) for the manufacture of the medicament.

Effective amounts of polypeptides for the foregoing methods are empirically determined using standard *in vitro* and *in vivo* dose-response assays. In addition, experimental data provided herein provide guidance as to amounts of polypeptides of the invention that are effective for achieving a desired biological response. For example, the dissociation constants determined for one form of mature VEGF-C ( $K_D=135$  pM for VEGFR-3 and  $K_D=410$  pM for VEGFR-2) provide an indication as to the concentration of VEGF-C necessary to achieve biological effects, because such dissociation constants represent concentrations at which half of the VEGF-C polypeptide is bound to the receptors through which VEGF-C biological effects are mediated. Results from *in vivo* Miles assays, wherein 0-8 picomoles of VEGF-C was injected intradermally, provide an indication that picomole quantities of mature VEGF-C are sufficient to induce localized biological effects. *In vitro* analysis of  $^3\text{H}$ -thymidine incorporation into bovine capillary endothelial cells treated with a mature VEGF-C form showed increasing VEGF-C effects on cell proliferation at concentrations of 10-1000 pM. Collectively, this data suggests that localized concentrations of 100-1000 pM of fully-processed VEGF-C have VEGF-C biological activity *in vivo*. Effective concentrations of other polypeptides of the invention are generally expected to correlate with the dissociation constant of the polypeptides for the relevant receptors. Pharmacokinetic and pharmacological analyses reveals the preferred dosages, dosage formulations, and methods of administration to achieve the desired local or systemic concentration of a polypeptide of the invention.

Polypeptides of the invention also may be used to quantify future metastatic risk by assaying biopsy material for the presence of active receptors or ligands in a binding assay. Such a binding assay may involve the use of a detectably labeled polypeptide of the invention or of an unlabeled polypeptide in conjunction with a labeled antibody, for example. Kits comprising such substances are included within the scope of the invention.

The present invention also provides methods for using the claimed nucleic acids (i.e., polynucleotides) in screening for endothelial cell disorders. In a preferred embodiment, the invention provides a method for screening an endothelial cell disorder in a mammalian subject comprising the steps of providing a sample of endothelial cell nucleic acids from the subject, contacting the sample of endothelial cell nucleic acids with a polynucleotide of the invention which is capable of hybridizing to a gene encoding VEGF-C (and preferably capable of hybridizing to VEGF-C mRNA), determining the level of hybridization between the endothelial cell nucleic acids and the polynucleotide, and corre-

lating the level of hybridization with a disorder. A preferred mammalian subject, and source of endothelial cell nucleic acids, is a human. The disorders contemplated by the method of screening with polynucleotides include, but are not limited to, vessel disorders such as the aforementioned lymphatic vessel disorders, and hypoxia.

Purified and isolated polynucleotides encoding other (non-human) VEGF-C forms also are aspects of the invention, as are the polypeptides encoded thereby, and antibodies that are specifically immunoreactive with the non-human VEGF-C forms. Preferred non-human forms of VEGF-C are forms derived from other vertebrate species, including avian and mammalian species. Mammalian forms are highly preferred. Thus, the invention includes a purified and isolated mammalian VEGF-C polypeptide, and also a purified and isolated polynucleotide encoding such a polypeptide.

In one embodiment, the invention includes a purified and isolated polypeptide having the amino acid sequence of residues 1 to 415 of SEQ ID NO: 11, which sequence corresponds to a putative mouse VEGF-C precursor. The putative mouse VEGF-C precursor is believed to be processed into a mature mouse VEGF-C in a manner analogous to the processing of the human prepro-polypeptide. Thus, in a related aspect, the invention includes a purified and isolated polypeptide capable of specifically binding to an Flt4 receptor tyrosine kinase (e.g., a human or mouse Flt4 receptor tyrosine kinase), the polypeptide comprising a fragment of the purified and isolated polypeptide having the amino acid sequence of residues 1 to 415 of SEQ ID NO: 11, the fragment being capable of specifically binding to the Flt4 receptor tyrosine kinase. The invention further includes multimers of the foregoing polypeptides and purified and isolated nucleic acids encoding the foregoing polypeptides, such as a nucleic acid comprising all or a portion of the sequence shown in SEQ ID NO: 10.

In another embodiment, the invention includes a purified and isolated quail VEGF-C polypeptide, biologically active fragments and multimers thereof, and polynucleotides encoding the foregoing polypeptides.

It is also contemplated that VEGF-C polypeptides from other species may be altered in the manner described herein with respect to human VEGF-C variants, in order to alter biological properties of the wildtype protein. For example, elimination of the cysteine at position 152 of SEQ ID NO: 11 or position 155 of SEQ ID NO: 13 is expected to alter VEGFR-2 binding properties in the manner described below for human VEGF-C  $\Delta C_{155}$  mutants.

In yet another embodiment, the invention includes a DNA comprising a VEGF-C promoter, that is capable of promoting expression of a VEGF-C gene or another operatively-linked, protein-encoding gene in native host cells, under conditions wherein VEGF-C is expressed in such cells. Thus, the invention includes a purified nucleic acid comprising a VEGF-C promoter sequence. Genomic clone lambda 5 described herein comprises more than 5 kb of human genomic DNA upstream of the VEGF-C translation initiation codon, and contains promoter DNA of the invention. Approximately 2.4 kb of this upstream sequence is set forth in SEQ ID NO: 48. Thus, in one embodiment, the invention includes a purified nucleic acid comprising a portion of SEQ ID NO: 48, wherein the portion is capable of promoting expression of a protein encoding gene operatively linked thereto under conditions wherein VEGF-C is expressed in native host cells. Similarly, the invention includes a chimeric nucleic acid comprising a VEGF-C

promoter nucleic acid according to the invention operatively connected to a sequence encoding a protein other than a human VEGF-C.

Additional aspects and embodiments of the invention will be apparent from the detailed description which follows.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 schematically depicts major endothelial cell receptor tyrosine kinases and growth factors involved in vasculogenesis and angiogenesis. Major structural domains are depicted, including immunoglobulin-like domains (IGH), epidermal growth factor homology domains (EGFH), fibronectin type III domains (FNIII), transmembrane (TM) and juxtamembrane (JM) domains, tyrosine kinase (TK1, TK2) domains, kinase insert domains (KI), and carboxy-terminal domains (CT).

FIGS. 2A, 2B, and 2C show a comparison of the deduced amino acid sequences of PDGF-A (SEQ ID NO: 53), PDGF-B (SEQ ID NO: 54), PIGF-1 (SEQ ID NO: 55), VEGF-B<sub>167</sub> (SEQ ID NO: 56), VEGF165 (SEQ ID NO: 57), and Flt4 ligand (VEGF-C, (SEQ ID NO: 8)).

FIG. 3A is an autoradiograph showing recombinant VEGF-C isolated following a pulse-chase experiment and electrophoresed via SDS-PAGE under reducing conditions.

FIG. 3B is a photograph of polyacrylamide gel showing that recombinant VEGF-C forms are disulfide-linked in nonreducing conditions.

FIG. 4 shows that VEGF-C stimulates endothelial cell migration in a three-dimensional collagen gel assay.

FIG. 5 depicts the amino acid sequences of human (SEQ ID NO: 8), murine (SEQ ID NO: 11), and quail (SEQ ID NO: 13) VEGF-C polypeptides, aligned to show similarity. Residues conserved in all three species are depicted in bold.

FIGS. 6A-C depict electrophoretic fractionations of the various forms of recombinant VEGF-C produced by transfected 293 EBNA cells. FIG. 6B depicts the electrophoretic fractionation, under non-reducing conditions, of polypeptides produced from mock (M) transfected cells, cells transfected with wild type (wt) VEGF-C cDNA, and cells transfected with a cDNA encoding the VEGF-C mutant VEGF-C-R102S. Each of the bands identified in FIG. 6B was excised and electrophoretically fractionated in a separate lane under reducing conditions. Fractionation of bands corresponding to wt VEGF-C are depicted in FIG. 6A; fractionation of bands corresponding to the R102S mutant are depicted in FIG. 6C.

FIGS. 7A-B depict the forms and sizes of wild type and mutant recombinant VEGF-Cs, as revealed by non-reducing gel electrophoresis. FIG. 7A shows the VEGF-C forms secreted into the media; FIG. 7B shows the VEGF-C forms retained by the cells. Mock (M) transfected cells served as a control.

FIGS. 8A-B present a comparison of the pattern of immunoprecipitated, labelled VEGF-C forms using antisera 882 and antisera 905. Adjacent lanes contain immunoprecipitates that were (lanes marked +) or were not (lanes marked -) subjected to reduction and alkylation.

FIG. 9 is a schematic model of the proteolytic processing of VEGF-C. The regions of the VEGF-C polypeptide are depicted as follows: signal sequence=dark shaded box; VEGF-homology domain=medium shaded box; N-terminal and C-terminal propeptides=dotted and open boxes, respectively. Conserved cysteine residues in the VEGF-homology domain are depicted with dots (for clarity, cysteine residues in the C-terminal propeptide are not marked). Putative sites

of N-linked glycosylation are shown with Y symbols. Numbers indicate approximate molecular mass (kDa) of the corresponding polypeptide as measured by SDS-PAGE in reducing conditions. Disulfide bonds are marked as —S—S—; non-covalent bonds are depicted as dotted lines. A question mark indicates the presence of a possible non-covalent bond. The proteolytic generation of a small fraction of disulfide-linked 21 kDa forms is not indicated in the figure. Several intermediate forms also are omitted to simplify the scheme. Particularly, only one precursor polypeptide is cleaved initially. The figure is not intended to suggest that other intermediate forms, for example 21 kDa+31 kDa, 31 kDa+31 kDa+29 kDa, do not exist.

FIG. 10 presents a comparison of the human and mouse VEGF-C amino acid sequences. The amino acid sequence of mouse VEGF-C is presented on the top line and differences in the human sequence are marked below it. An arrow indicates the putative cleavage site for the signal peptidase; BR3P motifs, as well as a CR/SC motif, are boxed; and conserved cysteine residues are marked in bold above the sequence. Arginine residue-158 is also marked in bold. The numbering refers to mouse VEGF-C residues.

FIGS. 11A-B present gel electrophoretograms of human VEGF-C (wt) and VEGF-C mutants secreted (FIG. 11A) or retained (FIG. 11B) by the host 293 EBNA cells. Mock (M) transfected cells served as a control. Molecular weight markers are indicated on the left in kilodaltons (kD).

FIG. 12 depicts the exon-intron organization of the human VEGF-C gene. Seven exons are depicted as open boxes, with exon size depicted in base pairs. Introns are depicted as lines, with intron size (base pairs) depicted above the lines. 5' and 3' untranslated sequences of a putative 2.4 kb mature mRNA are depicted as shaded boxes. The location of genomic clones used to characterize the VEGF-C gene are depicted below the map of the gene.

FIGS. 13A and 13B depict the genomic structure of the human (13A) and murine (13B) VEGF-C genes. Sequences of exon-intron junctions are depicted together with exon and intron lengths. Intron sequences are depicted in lower case letters. Nucleotides of the open reading frame observed in VEGF-C cDNAs are indicated as upper case letters in triplets (corresponding to the codons encoded at the junctions).

FIG. 14A depicts autoradiograms showing the binding of recombinantly produced wildtype VEGF-C (wt) and three VEGF-C mutants (VEGF-C R226,227S (R226,227S); VEGF-C ΔNΔCHis (Δ); and VEGF-C ΔNΔCHisC156S (Δ156S)) to a VEGFR-3 extracellular domain-immunoglobulin construct (left panel, VEGFR-3EC), to a VEGFR-2 extracellular domain-immunoglobulin construct (center panel, VEGFR-2EC), and to protein A sepharose (right panel, PAS).

FIG. 14B graphically depicts the results of a competitive binding assay. The ability of VEGF165, wildtype VEGF-C, and various VEGF-C mutants to compete with <sup>125</sup>I-VEGF-CΔNΔCHis for binding to VEGFR-2 and VEGFR-3 is shown.

FIG. 15 schematically depicts the VEGF-C promoter-reporter constructs and their activities in transfected HeLa cells. A restriction map of a portion of a genomic clone that includes the VEGF-C initiation codon and about 6 kb of upstream sequence is depicted above the constructs. Constructs were made linking putative VEGF-C promoter to the Luciferase reporter gene in pGL3 vector (Promega) and introduced into HeLa cells by calcium phosphate-mediated transfection method. The Luciferase activity obtained was



compared to the level using the promoterless pGL3basic construct to obtain a measure of relative promoter activity. Luciferase activity is expressed graphically as a ratio of activity of the constructs versus this control. Also shown are numerical ratios of Luciferase activity in experiments where the constructs were transfected into HeLa cells and cells were starved 24 hours followed by serum stimulation for four hours (Luciferase activity is expressed as a ratio of activity in serum-stimulated versus serum-starved cells).

#### DETAILED DESCRIPTION OF THE INVENTION

Described herein is the isolation of a novel vascular endothelial growth factor and the cloning of a DNA encoding this novel growth factor from a cDNA library prepared from the human prostatic adenocarcinoma cell line PC-3. The isolated cDNA encodes a protein which is proteolytically processed and secreted to cell culture medium. The processing is described in detail below. The secreted protein, designated VEGF-C, binds to the extracellular domain and induces tyrosine autophosphorylation of both Flt4 (VEGFR-3) and kdr/flk-1 (VEGFR-2). In contrast, neither VEGF nor PlGF showed specific binding to VEGFR-3 or induced its autophosphorylation. VEGF-C also stimulates the migration of endothelial cells in collagen gel and induces vascular permeability in vivo. In transgenic mice, VEGF-C induces proliferation of the lymphatic endothelium and causes an increase in neutrophilic granulocytes. Based on studies of VEGF-C variants and analogs and studies of VEGF precursors, it is anticipated that one or more VEGF-C precursors (the largest putative native VEGF-C precursor, excluding signal peptide, having the complete amino acid sequence from residue 32 to residue 419 of SEQ ID NO: 8) is capable of stimulating VEGFR-3.

In addition to providing a cDNA sequence encoding prepro-VEGF-C, the present application also provides significant guidance concerning portions of the VEGF-C amino acid sequence which are necessary for biological activity and portions (of one or more amino acids) which, when altered, will modulate (up-regulate or inhibit) VEGF-C biological activities. Such alterations are readily achieved through recombinant DNA and protein techniques, such as site-directed mutagenesis of a VEGF-C encoding cDNA and recombinant expression of the resultant modified cDNA. The skilled artisan also understands that, in recombinant production of proteins, additional sequence may be expressed along with a sequence encoding a polypeptide having a desired biological activity, while retaining a desired biological activity of the protein. For example, additional amino acids may be added at the amino terminus, at the carboxy-terminus, or as an insertion into the polypeptide sequence. Similarly, deletion variants of a protein with a desired biological activity can be recombinantly expressed that lack certain residues of the endogenous/natural protein, while retaining a desired biological activity. Moreover, it is well-known that recombinant protein variants may be produced having conservative amino acid replacements (including but not limited to substitution of one or more amino acids for other amino acids having similar chemical side-chains (acidic, basic, aliphatic, aliphatic hydroxyl, aromatic, amide, etc.)) which do not eliminate the desired biological activity of the protein. Accordingly, it is anticipated that such alterations of VEGF-C are VEGF-C equivalents within the scope of the invention.

As set forth in greater detail below, the putative prepro-VEGF-C has a deduced molecular mass of 46.883; a putative prepro-VEGF-C processing intermediate has an

observed molecular weight of about 32 kD; and mature VEGF-C isolated from conditioned media has a molecular weight of about 23 kD as assessed by SDS-PAGE under reducing conditions. A major part of the difference in the observed molecular mass of the purified and recombinant VEGF-C and the deduced molecular mass of the prepro-VEGF-C encoded by the "VEGF-C open reading frame (ORF)" is attributable to proteolytic removal of sequences at the amino-terminal and carboxyl-terminal regions of the prepro-VEGF-C polypeptide. Extrapolation from studies of the structure of PDGF (Heldin et al., *Growth Factors*, 8:245-52 (1993)) suggests that the region critical for receptor binding and activation by VEGF-C is contained within amino acids residues 104-213, which are found in the secreted form of the VEGF-C protein (i.e., the form lacking the putative prepro leader sequence and some carboxy-terminal sequences). The 23 kD polypeptide binding VEGFR-3 corresponds to a VEGF-homologous domain of VEGF-C. After biosynthesis, the nascent VEGF-C polypeptide may be glycosylated at three putative N-linked glycosylation sites identified in the deduced VEGF-C amino acid sequence. Polypeptides containing modifications, such as N-linked glycosylations, are intended as aspects of the invention.

The carboxyl terminal amino acid sequences, which increase the length of the VEGF-C polypeptide in comparison with other ligands of this family, show a pattern of spacing of cysteine residues reminiscent of the Balbiani ring 3 protein (BR3P) sequence (Dignam et al., *Gene*, 88:133-40 (1990); Paulsson et al., *J. Mol. Biol.*, 211:331-49 (1990)). This novel C-terminal silk protein-like structural motif of VEGF-C may fold into an independent domain, which is cleaved off after biosynthesis. Interestingly, at least one cysteine motif of the BR3P type is also found in the carboxyl terminus of VEGF. As explained in detail below, putative precursors and putative fully-processed VEGF-C were both detected in the cell culture media, suggesting cleavage by cellular proteases. The determination of amino-terminal and carboxy-terminal sequences of VEGF-C isolates was performed to identify the proteolytic processing sites. Antibodies generated against different parts of the pre-VEGF-C molecule were used to determine the precursor-product relationship and ratio, their cellular distribution, and the kinetics of processing and secretion.

VEGF-C has a conserved pattern of eight cysteine residues, which may participate in the formation of intra- and interchain disulfide bonds, creating an antiparallel, dimeric, biologically active molecule, similar to PDGF. Mutational analysis of the cysteine residues involved in the interchain disulfide bridges has shown that, in contrast to PDGF, VEGF dimers need to be held together by these covalent interactions in order to maintain biological activity. Disulfide linking of the VEGF-C polypeptide chains was evident in the analysis of VEGF-C in nonreducing conditions, although recombinant protein also contained "fully processed" ligand-active VEGF-C forms which lacked disulfide bonds between the polypeptides. (See FIG. 9.)

VEGFR-3, which distinguishes between VEGF and VEGF-C, is closely related in structure to VEGFR-1 and VEGFR-2. Finnerty et al., *Oncogene*, 8:2293-98 (1993); Galland et al., *Oncogene*, 8:1233-40 (1993); Pajusola et al., *Cancer Res.*, 52:5738-43 (1992). Besides VEGFR-3, VEGFR-2 tyrosine kinase also is activated in response to VEGF-C. VEGFR-2 mediated signals cause striking changes in the morphology, actin reorganization and membrane ruffling of porcine aortic endothelial cells overexpressing this receptor. In these cells, VEGFR-2 also medi-



ated ligand-induced chemotaxis and mitogenicity. Waltenberger et al., *J. Biol. Chem.*, 269:26988-95 (1994). Similarly, the receptor chimera CSF-1R/VEGFR-3 was mitogenic when ectopically expressed in NIH 3T3 fibroblastic cells, but not in porcine aortic endothelial cells (Pajusola et al., 1994). Consistent with such results, the bovine capillary endothelial (BCE) cells, which express VEGFR-2 mRNA but very little or no VEGFR-1 or VEGFR-3 mRNAs, showed enhanced migration when stimulated with VEGF-C. Light microscopy of the BCE cell cultures in collagen gel also suggested that VEGF-C stimulated the proliferation of these cells. The data thus indicate that the VEGF family of ligands and receptors show a great specificity in their signalling, which may be cell-type-dependent.

The expression pattern of the VEGFR-3 (Kaipainen et al., *Proc. Natl. Acad. Sci. (USA)*, 92:3566-70 (1995)) suggests that VEGF-C may function in the formation of the venous and lymphatic vascular systems during embryogenesis. Constitutive expression of VEGF-C in adult tissues shown herein further suggests that this gene product also is involved in the maintenance of the differentiated functions of the lymphatic and certain venous endothelia where VEGFR-3 is expressed (Kaipainen et al., 1995). Lymphatic capillaries do not have well-formed basal laminae and an interesting possibility exists that the silk-like BR3P motif is involved in producing a supramolecular structure which could regulate the availability of VEGF-C in tissues. However, as shown here, VEGF-C also activates VEGFR-2, which is abundant in proliferating endothelial cells of vascular sprouts and branching vessels of embryonic tissues, but not so abundant in adult tissues. Millauer et al., *Nature*, 367:576-78 (1993). These data have suggested that VEGFR-2 is a major regulator of vasculogenesis and angiogenesis. VEGF-C may thus have a unique effect on lymphatic endothelium and a more redundant function, shared with VEGF, in angiogenesis and possibly in regulating the permeability of several types of endothelia. Because VEGF-C stimulates VEGFR-2 and promotes endothelial migration, VEGF-C may be useful as an inducer of angiogenesis of blood and lymphatic vessels in wound healing, in tissue transplantation, in eye diseases, and in the formation of collateral vessels around arterial stenoses and into injured tissues after infarction.

Previously-identified growth factors that promote angiogenesis include the fibroblast growth factors, hepatocyte growth factor/scatter factor, PDGF and TGF- $\alpha$ . (See e.g., Folkman, *Nature Med.*, 1:27-31 (1995); Friesel et al., *FASEB J.*, 9:919-25 (1995); Mustonen et al., *J. Cell. Biol.*, 129:895-98 (1995). However, VEGF has been the only growth factor relatively specific for endothelial cells. The newly identified factors VEGF-B [Olofsson et al., *Proc. Natl. Acad. Sci.*, 93:2578-81 (1996)] and VEGF-C thus increase our understanding of the complexity of the specific and redundant positive signals for endothelial cells involved in vasculogenesis, angiogenesis, permeability, and perhaps also other endothelial functions. Expression studies using Northern blotting show abundant VEGF-C expression in heart and skeletal muscle; other tissues, such as placenta, ovary, small intestine, thyroid gland, kidney, prostate, spleen, testis and large intestine also express this gene. Whereas PlGF is predominantly expressed in the placenta, the expression patterns of VEGF, VEGF-B and VEGF-C overlap in many tissues, which suggests that members of the VEGF family may form heterodimers and interact to exert their physiological functions.

Targeted mutagenesis leading to inactivation of the VEGF receptor loci in the mouse genome has shown that VEGFR-1

is necessary for the proper organization of endothelial cells forming the vascular endothelium, while VEGFR-2 is necessary for the generation of both endothelial and hematopoietic cells. This suggests that the four genes of the VEGF family can be targets for mutations leading to vascular malformations or cardiovascular diseases.

The following Examples illustrate preferred embodiments of the invention, wherein the isolation, characterization, and function of VEGF-C, VEGF-C variants and analogs, VEGF-C-encoding nucleic acids, and anti-VEGF-C antibodies according to the invention are shown.

#### EXAMPLE 1

##### Production of pLTRFlt4l Expression Vector

The identification and isolation of two forms of Flt4 receptor tyrosine kinase (VEGFR-3) cDNA (Flt4 short form (Flt4s), Genbank Accession No. X68203, SEQ ID NO: 1; and Flt4 long form, (Flt4l), Genbank Accession Nos. X68203 and S66407, SEQ ID NO: 2) was reported in U.S. patent application Ser. No. 08/340,011, filed Nov. 14, 1994, incorporated by reference herein. An Flt4 expression vector designated pLTRFlt4l (encoding the long form of Flt4) was constructed using the pLTRpoly expression vector reported in Mäkelä et al., *Gene*, 118: 293-294 (1992) (Genbank accession number X60280, SEQ ID NO: 3) and the Flt4 cDNAs, in the manner described in commonly-owned PCT patent application PCT/FI96/00427, filed Aug. 1, 1996, and commonly-owned U.S. patent application Ser. Nos. 08/671,573, filed Jun. 28, 1996; 08/601,132, filed Feb. 14, 1996; 08/585,895, filed Jan. 12, 1996; and 08/510,133, filed Aug. 1, 1995, all of which are incorporated by reference in their entirety.

#### EXAMPLE 2

##### Production and Analysis of Flt4l Transfected Cells

NIH 3T3 cells (60% confluent) were co-transfected with 5 micrograms of the pLTRFlt4l construct and 0.25 micrograms of the pSV2neo vector containing the neomycin phosphotransferase gene (Southern et al., *J. Mol. Appl. Genet.*, 1:327 (1982)), using the DOTAP liposome-based transfection reagents (Boehringer-Mannheim, Mannheim, Germany). One day after transfection, the cells were transferred into selection media containing 0.5 mg/ml geneticin (GIBCO, Grand Island, N.Y.). Colonies of geneticin-resistant cells were isolated and analyzed for expression of the Flt4 proteins. Cells were lysed in boiling lysis buffer containing 3.3% SDS and 125 mM Tris, pH 6.8. Protein concentrations of the samples were measured by the BCA method (Pierce, Rockford, Ill.). About 50 micrograms of protein from each lysate were analyzed for the presence of Flt4 by 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using antisera against the carboxyl terminus of Flt4. Signals on Western blots were revealed using the ECL method (Amersham).

For production of anti-Flt4 antiserum, the Flt4 cDNA fragment encoding the 40 carboxy-terminal amino acid residues of the Flt4 short form: NH<sub>2</sub>-PMTPTTYKG SVD-NQTDSGM VLASEEFEQI ESRHRQESGFR-COOH (SEQ ID NO: 4) was cloned as a 657 bp EcoRI-fragment into the pGEX-11.T bacterial expression vector (Pharmacia-LKB, Inc., Uppsala, Sweden) in frame with the glutathione-S-transferase coding region. The resultant GST-Flt4S fusion protein was produced in *E. coli* and purified by affinity chromatography using a glutathione-Sepharose 4B column.

The purified protein was lyophilized, dissolved in phosphate-buffered saline (PBS), mixed with Freund's adjuvant and used for immunization of rabbits at bi-weekly intervals using methods standard in the art (Harlow et al., *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1988)). Antisera were used, after the fourth booster immunization, for immunoprecipitation of Flt4 from transfected cells. Cell clones expressing Flt4 were also used for ligand stimulation analysis.

#### EXAMPLE 3

##### Construction of a Flt4 EC Baculovirus Vector and Expression and Purification of its Product

Using the pVTBac plasmid described in Tessier et al., *Gene* 98:177-183 (1991), and the Flt4 cDNAs described in Example 1, a baculovirus expression vector was constructed to facilitate expression of the extracellular domain of Flt4 (Flt4 EC), as described in commonly-owned PCT patent application PCT/FI96/00427, filed Aug. 1, 1996, and commonly-owned U.S. patent application Ser. Nos. 08/671,573, filed Jun. 28, 1996; 08/601,132, filed Feb. 14, 1996; 08/585,895, filed Jan. 12, 1996; and 08/510,133, filed Aug. 1, 1995, all of which are incorporated by reference herein. A nucleotide sequence encoding a 6xHis tag was operatively connected to the Flt4 EC coding sequence to facilitate purification.

The Flt4EC construct was transfected together with baculovirus genomic DNA into SF-9 cells by lipofection. Recombinant virus was purified, amplified and used for infection of High-Five cells (Invitrogen, San Diego, Calif.) using methods standard in the art. The Flt4 extracellular domain (Flt4EC) was purified from the culture medium of the infected High-Five cells using Ni-NTA affinity chromatography according to manufacturer's instructions (Qiagen) for binding and elution of the 6xHis tag encoded in the COOH-terminus of the recombinant Flt4 extracellular domain.

#### EXAMPLE 4

##### Isolation of an Flt4 Ligand from Conditioned Media

A human Flt4 ligand according to the invention was isolated from media conditioned by a PC-3 prostatic adenocarcinoma cell line (ATCC CRL 1435) in serum-free Ham's F-12 Nutrient mixture (GIBCO) (containing 7% fetal calf serum (FCS)). Cells were reseeded and grown in this medium, which was subsequently changed to serum-free medium. The preparation of the conditioned media, and the identification of a component therein which stimulated Flt4 tyrosine phosphorylation, are described in detail in commonly-owned PCT patent application PCT/FI96/00427, filed Aug. 1, 1996, and commonly-owned U.S. patent application Ser. Nos. 08/671,573, filed Jun. 28, 1996; 08/601,132, filed Feb. 14, 1996; 08/585,895, filed Jan. 12, 1996; 08/510,133, filed Aug. 1, 1995; and 08/340,011, filed Nov. 14, 1994 now U.S. Pat. No. 5,726,755, all of which are incorporated by reference herein in their entirety. The ability of the conditioned medium to stimulate Flt4 phosphorylation was considerably increased when the PC-3 conditioned medium was concentrated four-fold using a Centricon-10 concentrator (Amicon). Pretreatment of the concentrated PC-3 conditioned medium with 50 microliters of Flt4 extracellular domain coupled to CNBr-activated sepharose CL-4B (Pharmacia; about 1mg of Flt4EC domain/ml sepharose

resin) completely abolished Flt4 tyrosine phosphorylation. Similar pretreatment of the conditioned medium with unsubstituted sepharose CL-4B did not affect stimulatory activity. Also, the flow through obtained after concentration, which contained proteins of less than 10,000 molecular weight, did not stimulate Flt4 phosphorylation.

In another experiment, a comparison of Flt4 autophosphorylation in transformed NIH 3T3 cells expressing LTRFlt4I was conducted, using unconditioned medium, medium from PC-3 cells expressing the Flt4 ligand, or unconditioned medium containing either 50 ng/ml of VEGF165 or 50 ng/ml of PlGF-1. The cells were lysed, immunoprecipitated using anti-Flt4 antiserum and analyzed by Western blotting using anti-phosphotyrosine antibodies. Only the PC-3 conditioned medium expressing the Flt4 ligand (lane Flt4-L) stimulated Flt4 autophosphorylation.

These experiments showed that PC-3 cells produce a ligand which binds to the extracellular domain of Flt4 and activates this receptor.

#### EXAMPLE 5

##### Purification of the Flt4 Ligand

The ligand expressed by human PC-3 cells as characterized in Example 4 was purified and isolated using a recombinantly-produced Flt4 extracellular domain (Flt4EC) in affinity chromatography.

Two harvests of serum-free conditioned medium, comprising a total of 8 liters, were collected from 500 confluent 15 cm diameter culture dishes containing confluent layers of PC-3 cells. The conditioned medium was clarified by centrifugation at 10,000xg and concentrated 80-fold using an Ultrascetic Tangential Flow Device (Filtron, Northborough, Mass.) with a 10 kD cutoff Omega Ultrafiltration membrane according to the manufacturer's instructions. Recombinant Flt4 extracellular domain was expressed in a recombinant baculovirus cell system and purified by affinity chromatography on Ni-agarose (Ni-NTA affinity column obtained from Qiagen). The purified extracellular domain was coupled to CNBr-activated Sepharose CL-4B at a concentration of 5 mg/ml and used as an affinity matrix for ligand affinity chromatography.

Concentrated conditioned medium was incubated with 2 ml of the recombinant Flt4 extracellular domain-Sepharose affinity matrix in a rolling tube at room temperature for 3 hours. All subsequent purification steps were at +4° C. The affinity matrix was then transferred to a column with an inner diameter of 15 mm and washed successively with 100 ml of PBS and 50 ml of 10 mM Na-phosphate buffer (pH 6.8). Bound material was eluted step-wise with 100 mM glycine-HCl, successive 6 ml elutions having pHs of 4.0, 2.4, and 1.9. Several 2 ml fractions of the eluate were collected in tubes containing 0.5 ml 1 M Na-phosphate (pH 8.0). Fractions were mixed immediately and dialyzed in 1 mM Tris-HCl (pH 7.5). Aliquots of 75 µl each were analyzed for their ability to stimulate tyrosine phosphorylation of Flt4. The ultrafiltrate, 100 µl aliquots of the concentrated conditioned medium before and after ligand affinity chromatography, as well as 15-fold concentrated fractions of material released from the Flt4 extracellular domain-Sepharose matrix during the washings were also analyzed for their ability to stimulate Flt4 tyrosine phosphorylation.

The concentrated conditioned medium induced prominent tyrosine phosphorylation of Flt4 in transfected NIH 3T3 cells overexpressing Flt4. This activity was not observed in conditioned medium taken after medium was exposed to the

Flt4 Sepharose affinity matrix. The specifically-bound Flt4-stimulating material was retained on the affinity matrix after washing in PBS, 10 mM Na-phosphate buffer (pH 6.8), and at pH 4.0. It was eluted in the first two 2 ml aliquots at pH 2.4. A further decrease of the pH of the elution buffer did not cause release of additional Flt4-stimulating material. No Flt4 phosphorylation was observed in a control wherein Flt4-expressing cells were treated with unconditioned medium; similarly, no phosphorylation was observed following treatment of Flt4-expressing cells with the ultrafiltrate fraction of conditioned medium containing polypeptides of less than 10 kD molecular weight.

Small aliquots of the chromatographic fractions were concentrated in a SpeedVac concentrator (Savant, Farmingdale, N.Y.) and subjected to SDS-PAGE under reducing conditions with subsequent silver staining of the gel, a standard technique in the art. The major polypeptide, having a molecular weight of approximately 23 kD (reducing conditions), was detected in the fractions containing Flt4 stimulating activity. That polypeptide was not found in the other chromatographic fractions. On the other hand, besides these bands and a very faint band having a 32 kD mobility, all other components detected in the two active fractions were also distributed in the starting material and in small amounts in the other washing and eluting steps after their concentration. Similar results were obtained in three independent affinity purifications, indicating that the 23 kD polypeptide specifically binds to Flt4 and induces tyrosine phosphorylation of Flt4.

Fractions containing the 23 kD polypeptide were combined, dried in a SpeedVac concentrator and subjected to SDS-PAGE in a 12.5% gel. The proteins from the gel were then electroblotted to Immobilon-P (PVDF) transfer membrane (Millipore, Marlborough, Mass.) and visualized by staining of the blot with Coomassie Blue R-250. The region containing only the stained 23 kD band was cut from the blot and subjected to N-terminal amino acid sequence analysis in a Prosite Protein Sequencing System (Applied Biosystems, Foster City, Calif.). The data were analyzed using a 610A Data Analysis System (Applied Biosystems). Analysis revealed a single N-terminal sequence of NH<sub>2</sub>-XEETIKFAAAHYNTEILK-COOH (SEQ ID NO: 5).

#### EXAMPLE 6

##### Construction of PC-3 Cell cDNA Library in a Eukaryotic Expression Vector

Human poly(A)<sup>+</sup> RNA was isolated from five 15 cm diameter dishes of confluent PC-3 cells by a single step method using oligo(dT) (Type III, Collaborative Biomedical Products, Becton-Dickinson Labware, Bedford, Mass.) cellulose affinity chromatography (Sambrook et al., 1989). The yield was 70 micrograms. Six micrograms of the Poly(A)<sup>+</sup> RNA were used to prepare an oligo(dT)-primed cDNA library in the mammalian expression vector pcDNA 1 and the Librarian kit of Invitrogen according to the instructions included in the kit. The library was estimated to contain about 10<sup>6</sup> independent recombinants with an average insert size of approximately 1.8 kb.

#### EXAMPLES 7-9

##### Amplification of a cDNA Encoding the Flt4 Ligand Amino Terminus

The procedures used to isolate a cDNA encoding the Flt4 ligand are described in detail in commonly-owned PCT

patent application PCT/EP96/00427, filed Aug. 1, 1996, and commonly-owned U.S. patent application Ser. Nos. 08/671,573; filed Jun. 28, 1996; 08/601,132, filed Feb. 14, 1996; 08/585,895, filed Jan. 12, 1996; and 08/510,133, filed Aug. 1, 1995, all of which are incorporated by reference herein. Initially, degenerate oligonucleotides were designed based on the N-terminal amino acid sequence of the isolated human Flt4 ligand and were used as primers in a polymerase chain reaction (PCR) to amplify a partial cDNA encoding the (fully-processed) Flt4 ligand amino terminus from the PC-3 cDNA library. The amplified cDNA fragment was cloned into a pCR II vector (Invitrogen) using the TA cloning kit (Invitrogen) and sequenced using the radioactive dideoxynucleotide sequencing method of Sanger. Six clones were analyzed and all six clones contained the sequence encoding the expected peptide (amino acid residues 104-120 of the Flt4 ligand precursor, SEQ ID NO: 8). Nucleotide sequence spanning the region from the third nucleotide of codon 6 to the third nucleotide of codon 13 (the extension region between the PCR primers) was identical in all six clones and thus represented an amplified product from the unique sequence encoding part of the amino terminus of the Flt4 ligand.

Based on the unique nucleotide sequence encoding the N-terminus of the isolated human Flt4 ligand, two pairs of nested primers were designed to amplify, in two nested PCR reactions, the complete 5'-end of the corresponding cDNAs from one microgram of DNA of the above-described PC-3 cDNA library. One major product of about 220 bp and three minor products of about 270 bp, 150 bp, and 100 bp were obtained.

The amplified fragment of approximately 220 bp was excised from an agarose gel, cloned into a pCRII vector using the TA cloning kit, and sequenced. Three recombinant clones were analyzed and they contained the sequence 5'-TCACTATAGGGAGACCCAAGCTTGTTACCGAGCTCGGATCCACIAGTAAACGGCCGCC AGTGTGGTGGAATTCGACGAACCTCATGA-CTGTACTCTACCCAGAATATGGAAAATGTACAAGTGTCTAGCTAAGGCCAAGGAGGCTGGCAACATAACAGAGAACAGGCCAACCTCAACTCAAGGACAGAAAGAGACTATAAAATTCGCTGCAGCACACTACAAC-3' (SEQ ID NO: 6). The beginning of the sequence represents the vector and the underlined sequence represents the amplified product of the 5'-end of the cDNA insert.

Based upon the amplified 5'-sequence of the clones encoding the amino terminus of the 23 kD human Flt4 ligand, two pairs of non-overlapping nested primers were designed to amplify the 3'-portion of the Flt4-ligand-encoding cDNA clones via PCR. Two DNA fragments were obtained, having sizes of 1350 bp and 570 bp. Those fragments were cloned into a pCRII vector and the inserts of the clones were sequenced. Both of these fragments were found to contain sequences encoding an amino acid sequence homologous to the VEGF sequence.

#### EXAMPLE 10

##### Screening the PC-3 Cell cDNA Library Using the 5' PCR Fragment of Flt4 Ligand cDNA

A 153 bp fragment encoding the 5' end of the Flt4 ligand was labeled with [<sup>32</sup>P]-dCTP using the Klenow fragment of *E. coli* DNA polymerase I (Boehringer Mannheim). That fragment was used as a probe for hybridization screening of the amplified PC-3 cell cDNA library.

Filter replicas of the library were hybridized with the radioactively labeled probe at 42° C. for 20 hours in a

solution containing 50% formamide, 5xSSPE, 5xDenhardt's solution, 0.1% SDS and 0.1 mg/ml denatured salmon sperm DNA. Filters were washed twice in 1xSSC, 0.1% SDS for 30 minutes at room temperature, then twice for 30 minutes at 65° C. and exposed overnight.

On the basis of autoradiography, 10 positive recombinant bacterial colonies hybridizing with the probe were chosen from the library. Plasmid DNA was purified from these colonies and analyzed by EcoRI and NotI digestion and agarose gel electrophoresis followed by ethidium bromide staining. The ten plasmid clones were divided into three groups on the basis of the presence of insert sizes of approximately 1.7, 1.9 and 2.1 kb, respectively. Inserts of plasmids from each group were sequenced using the T7 oligonucleotide as a primer and walking primers for subsequent sequencing reactions.

Sequence analysis showed that all clones contain the open reading frame encoding the NH2-terminal sequence of the 23 kD human Flt4 ligand. Dideoxy sequencing was continued using walking primers in the downstream direction. A complete human cDNA sequence and deduced amino acid sequence from a 2 kb clone is set forth in SEQ ID NOs: 7 and 8, respectively. A putative cleavage site of a "prepro" leader sequence is located between residues 102 and 103 of SEQ ID NO: 8. When compared with sequences in the GenBank Database, the predicted protein product of this reading frame was found to be homologous with the predicted amino acid sequences of the PDGF/VEGF family of growth factors, as shown in FIGS. 2A, 2B and 2C.

Plasmid pFLT4-L, containing the 2.1 kb human cDNA clone in pcDNA1 vector, has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852 as accession number 97231.

#### EXAMPLE 11

##### Stimulation of Flt4 Autophosphorylation by the Protein Product of the Flt4 Ligand Vector

The 2.1 kb human cDNA insert of plasmid pFlt4-L, which contains the open reading frame encoding the sequence shown in SEQ ID NOs: 7 and 8; human prepro-VEGF-C, see below), was cut out from the pcDNA1 vector using HindIII and NotI restriction enzymes, isolated from a preparative agarose gel, and ligated to the corresponding sites in the pREP7 expression vector (Invitrogen). The pREP7 vector containing the pFlt4-L insert was transfected into 293-EBNA cells (Invitrogen) using the calcium phosphate transfection method (Sambrook et al., 1989). About 48 hours after transfection the medium of the transfected cells was changed to DMEM medium lacking fetal calf serum and incubated for 36 h. The conditioned medium was then collected, centrifuged at 5000xg for 20 minutes, the supernatant was concentrated 5-fold using Centriprep 10 (Amicon) and used to stimulate NIH 3T3 cells expressing LTRFlt41 (the Flt4 receptor) as in Example 4. The cells were lysed, immunoprecipitated using anti-Flt4 antiserum and analyzed by Western blotting using anti-phosphotyrosine antibodies.

The conditioned medium from two different dishes of the transfected cells stimulated Flt4 autophosphorylation in comparison with the medium from mock-transfected cells, which gave only background levels of phosphorylation of the Flt4 receptor. When the concentrated conditioned medium was pre-absorbed with 20 microliters of a slurry of Flt4EC domain coupled to Sepharose (see example 4), no phosphorylation was obtained, showing that the activity

responsible for Flt4 autophosphorylation was indeed the Flt4 ligand. Thus, these results demonstrate that an expression vector having an approximately 2.1 kb insert and containing an open reading frame as shown in SEQ ID NO: 7 is expressed as a biologically active Flt4 ligand (VEGF-C) in transfected cells. The sequence encoded by that open reading frame is shown in SEQ ID NO: 8.

The deduced molecular weight of a polypeptide consisting of the complete amino acid sequence in SEQ ID NO: 8 (residues 1 to 419) is 46,883. The deduced molecular weight of a polypeptide consisting of amino acid residues 103 to 419 of SEQ ID NO: 8 is 35,881. The Flt4 ligand purified from PC-3 cultures had an observed molecular weight of about 23 kD as assessed by SDS-PAGE under reducing conditions. Thus, it appeared that the Flt4 ligand mRNA was translated into a precursor polypeptide, from which the mature ligand was derived by proteolytic cleavage. Also, the Flt4 ligand may be glycosylated at three putative N-linked glycosylation sites conforming to the consensus which can be identified in the deduced Flt4 ligand amino acid sequence (N-residues underlined in FIGS. 2A, 2B and 2C).

The carboxyl terminal amino acid sequences, which increase the predicted molecular weight of the Flt4 ligand subunit in comparison with other ligands of this family, show a pattern of spacing of cysteine residues reminiscent of the Balbiani ring 3 protein (BR3P) sequence (Dignam et al., *Gene*, 88:133-140 (1990)). Such a sequence may encode an independently folded domain present in a Flt4 ligand precursor and it may be involved, for example, in the regulation of secretion, solubility, stability, cell surface localization or activity of the Flt4 ligand. Interestingly, at least one cysteine motif of the BR3P type is also found in the VEGF carboxy terminal amino acid sequences.

Thus, the Flt4 ligand mRNA appears first to be translated into a precursor from the mRNA corresponding to the cDNA insert of plasmid FLT4-L, from which the mature ligand is derived by proteolytic cleavage. To define the mature Flt4 ligand polypeptide, one first expresses the cDNA clone (which is deposited in the pcDNA1 expression vector) in cells, such as COS cells. One uses antibodies generated against encoded polypeptides, fragments thereof, or bacterial Flt4 fusion proteins, such as a GST-fusion protein, to raise antibodies against the VEGF-homologous domain and the amino- and carboxyl-terminal propeptides of Flt4 ligand. One then follows the biosynthesis and processing of the Flt4 ligand in the transfected cells by pulse-chase analysis using radioactive cysteine for labelling of the cells, immunoprecipitation, and gel electrophoresis. Using antibodies against the three domains of the product encoded by the cDNA insert of plasmid FLT4-L, material for radioactive or nonradioactive amino-terminal sequence analysis is isolated. The determination of the amino-terminal sequence of the mature VEGF-C polypeptide allows for identification of the amino-terminal proteolytic processing site. The determination of the amino-terminal sequence of the carboxyl-terminal propeptide will give the carboxyl-terminal processing site. This is confirmed by site-directed mutagenesis of the amino acid residues adjacent to the cleavage sites, which would prevent the cleavage.

The Flt4 ligand is further characterizable by progressive 3' deletions in the 3' coding sequences of the Flt4 ligand precursor clone, introducing a stop codon resulting in carboxy-terminal truncations of its protein product. The activities of such truncated forms are assayed by, for example, studying Flt4 autophosphorylation induced by the truncated proteins when applied to cultures of cells, such as NIH 3T3 cells expressing LTRFlt41. By extrapolation from

studies of the structure of the related platelet derived growth factor (PDGF, Heldin et al., *Growth Factors*, 8:245-252 (1993)) one determines that the region critical for receptor activation by the Flt4 ligand is contained within the first approximately 180 amino acid residues of the secreted VEGF-C protein lacking the putative 102 amino acid prepro leader (SEQ ID NO: 8, residues 103-282), and apparently within the first approximately 120 amino acid residues (SEQ ID NO: 8, residues 103-223).

On the other hand, the difference between the molecular weights observed for the purified ligand and deduced from the open reading frame of the Flt4 ligand clone may be due to the fact that the soluble ligand was produced from an alternatively spliced mRNA which would also be present in the PC-3 cells, from which the isolated ligand was derived. To isolate such alternative cDNA clones one uses cDNA fragments of the deposited clone and PCR primers made according to the sequence provided as well as techniques standard in the art to isolate or amplify alternative cDNAs from the PC-3 cell cDNA library. One may also amplify using reverse transcription (RT)-PCR directly from the PC-3 mRNA using the primers provided in the sequence of the CDNA insert of plasmid FLT4-L. Alternative cDNA sequences are determined from the resulting cDNA clones. One can also isolate genomic clones corresponding to the Flt4 ligand mRNA transcript from a human genomic DNA library using methods standard in the art and sequence such clones or their subcloned fragments to reveal the corresponding exons. Alternative exons can then be identified by a number of methods standard in the art, such as heteroduplex analysis of CDNA and genomic DNA, which are subsequently characterized.

#### EXAMPLE 12

##### Expression of the Gene Encoding VEGF-C in Human Tumor Cell Lines

Expression of transcripts corresponding to the Flt4 ligand (VEGF-C) was analyzed by hybridization of Northern blots containing isolated poly(A)<sup>+</sup> RNA from HT-1080 and PC-3 human tumor cell lines. The probe was the radioactively labelled insert of the 2.1 kb cDNA clone (pFlt4-L/VEGF-C, specific activity 10<sup>6</sup>-10<sup>7</sup> cpm/mg of DNA). The blot was hybridized overnight at 42° C. using 50% formamide, 5×SSPE buffer, 2% SDS, 10×Denhardt's solution, 100 mg/ml salmon sperm DNA and 1×10<sup>6</sup> cpm of the labelled probe/ml. The blot was washed at room temperature for 2×30 minutes in 2×SSC containing 0.05% SDS, and then for 2×20 minutes at 52° C. in 0.1×SSC containing 0.1% SDS. The blot was then exposed at -70° C. for three days using intensifying screens and Kodak XAR film. Both cell lines expressed an Flt4 ligand mRNA of about 2.4 kb, as well as VEGF and VEGF-B mRNAs.

#### EXAMPLE 13

##### VEGF-C Chains Are Proteolytically Processed After Biosynthesis and Disulfide Linked

The predicted molecular mass of a secreted human VEGF-C polypeptide, as deduced from the VEGF-C open reading frame, is 46.883 kD, suggesting that VEGF-C mRNA may be first translated into a precursor, from which the observed ligands of 21/23 kD and 29/32 kD are derived by proteolytic cleavage.

This possibility was explored by metabolic labelling of 293 EBNA cells expressing VEGF-C. Initially, 293 EBNA

cells were transfected with the VEGF-C cDNA construct. Expression products were labeled by the addition of 100  $\mu$ Ci/ml of Pro-mix<sup>TM</sup> L-[<sup>35</sup>S] in vitro cell labelling mix ((containing <sup>35</sup>S-methionine and <sup>35</sup>S-cysteine) Amersham, Buckinghamshire, England) to the culture medium devoid of cysteine and methionine. After two hours, the cell layers were washed twice with PBS and the medium was then replaced with DMEM-0.2% BSA. After 1, 3, 6, 12 and 24 hours of subsequent incubation, the culture medium was collected, clarified by centrifugation, and concentrated, and human VEGF-C was bound to 30  $\mu$ l of a slurry of Flt4EC-Sepharose overnight at +4° C., followed by three washes in PBS, two washes in 20 mM Tris-HCl (pH 7.5), alkylation, SDS-PAGE and autoradiography. Alkylation was carried out by treatment of the samples with 10 mM 1,4 Dithiothreitol (Boehringer-Mannheim, Mannheim, Germany) for one hour at 25° C., and subsequently with 30 mM iodoacetamide (Fluka, Buchs, Switzerland).

These experiments demonstrated that a putative precursor polypeptide of 32 kD apparent molecular mass was bound to the Flt4EC affinity matrix from the conditioned medium of metabolically labelled cells transfected with the human VEGF-C expression vector (FIG. 3A), but not from mock (M) transfected cells. Increased amounts of a 23 kD receptor binding polypeptide accumulated in the culture medium of VEGF-C transfected cells during a subsequent chase period of three hours, but not thereafter (lanes 2-4 and data not shown), suggesting that the 23 kD form is produced by proteolytic processing, which is incomplete, at least in the transiently transfected cells. The arrows in FIG. 3A indicate the 32 kD and 23 kD polypeptides of secreted VEGF-C. Subsequent experiments showed that the 32 kD VEGF-C form contains two components migrating in the absence of alkylation as polypeptides of 29 and 32 kD (FIGS. 6-8).

In a related experiment, human VEGF-C isolated using Flt4EC-Sepharose after a 4 h continuous metabolic labelling was analyzed by polyacrylamide gel electrophoresis in non-reducing conditions (FIG. 3B). Higher molecular mass forms were observed under nonreducing conditions, suggesting that the VEGF-C polypeptides can form disulfide-linked dimers and/or multimers (arrows in FIG. 3B).

Additional experiments have shown that higher molecular mass forms of VEGF-C (about 58 kD and about 43 kD) are observed under reducing conditions as well. (See below and FIGS. 6A and 14A.)

#### EXAMPLE 14

##### Stimulation Of VEGFR-2 Autophosphorylation By VEGF-C

Conditioned medium (CM) from 293 EBNA cells transfected with the human VEGF-C vector also was used to stimulate porcine aortic endothelial (PAE) cells expressing VEGFR-2 (Kdr). Pajusola et al., *Oncogene*, 9:3545-55 (1994); Waltenberger et al., *J. Biol. Chem.*, 269:26958-26995 (1994). The cells were lysed and immunoprecipitated using VEGFR-2-specific antiserum (Waltenberger et al., 1994).

PAE-KDR cells (Waltenberger et al., 1994) were grown in Ham's F12 medium-10% fetal calf serum (FCS). Confluent NIH 3T3-Flt4 cells or PAE-KDR cells were starved overnight in DMEM or Ham's F12 medium, respectively, supplemented with 0.2% bovine serum albumin (BSA), and then incubated for 5 minutes with the analyzed media. Recombinant human VEGF (R&D Systems) and PDGF-BB, functional as stimulating agents, were used as controls. The

cells were washed twice with ice-cold Tris-Buffered Saline (TBS) containing 100 mM sodium orthovanadate and lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 U/ml aprotinin and 1 mM sodium orthovanadate. The lysates were sonicated, clarified by centrifugation at 16,000xg for 20 minutes and incubated for 3-6 hours on ice with 3-5  $\mu$ l of antisera specific for Flt4 (Pajusola et al., 1993), VEGFR-2 or PDGFR- $\beta$  (Claesson-Welsh et al., *J. Biol. Chem.*, 264:1742-1747 (1989); Waltenberger et al., 1994). Immunoprecipitates were bound to protein A-Sepharose, washed three times with RIPA buffer containing 1 mM PMSF, 1 mM sodium orthovanadate, washed twice with 10 mM Tris-HCl (pH 7.4), and subjected to SDS-PAGE using a 7% gel. Polypeptides were transferred to nitrocellulose by Western blotting and analyzed using PY20 phosphotyrosine-specific monoclonal antibodies (Transduction Laboratories) or receptor-specific antiserum and the ECL detection method (Amersham Corp.).

PAE cells expressing VEGFR-2 were treated with 10- or 2-fold concentrated medium from mock-transfected 293-EBNA cells, or with 2-, 5- or 10-fold concentrated medium from 293-EBNA cell cultures expressing the recombinant VEGF-C. VEGFR-2 was immunoprecipitated with specific antibodies and analyzed by SDS-PAGE and Western blotting using phosphotyrosine antibodies. For comparison, the treatments were also carried out with non-conditioned medium containing 50 ng/ml of purified recombinant VEGF. Additional cells were also treated with VEGF-C- or VEGF-containing media pretreated with Flt4EC.

The results of this experiment were as follows. A basal level of tyrosine phosphorylation of VEGFR-2 was detected in cells stimulated by CM from the mock-transfected cells. A further concentration of this medium resulted in only a slight enhancement of VEGFR-2 phosphorylation. CM containing recombinant VEGF-C stimulated tyrosine autophosphorylation of VEGFR-2 and the intensity of the autophosphorylated polypeptide band was increased upon concentration of the VEGF-C CM. Furthermore, the stimulating effect was abolished after pretreatment of the medium with the Flt4EC affinity matrix. The maximal effect of VEGF-C in this assay was comparable to the effect of recombinant VEGF added to unconditioned medium at concentration of 50 ng/ml. Pretreatment of the medium containing VEGF with Flt4EC did not abolish its stimulating effect on VEGFR-2. These results suggest that the VEGF-C expression vector encodes a ligand not only for Flt4 (VEGFR-3), but also for Kdr/Flk-1 (VEGFR-2).

In order to further confirm that the stimulating effect of VEGF-C on tyrosine phosphorylation of VEGFR-3 and VEGFR-2 was receptor-specific, we analyzed the effect of VEGF-C on tyrosine phosphorylation of PDGF receptor  $\beta$  (PDGFR- $\beta$ ) which is abundantly expressed on fibroblastic cells. PDGFR- $\beta$ -expressing NIH 3T3 cells were treated with non-conditioned medium, 5-fold concentrated CM from mock-transfected or VEGF-C-transfected cells, or with non-conditioned medium containing 50 ng/ml of recombinant human PDGF-BB. Medium containing VEGF-C was also pretreated with recombinant Flt4EC (lane 4). PDGFR- $\beta$  was immunoprecipitated with specific antibodies and analyzed by SDS-PAGE and Western blotting using phosphotyrosine antibodies with subsequent stripping and reprobing of the membrane with antibodies specific for PDGFR- $\beta$ . A weak tyrosine phosphorylation of PDGFR- $\beta$  was detected upon stimulation of Flt4-expressing NIH 3T3 cells with CM from the mock-transfected cells. A similar low level of PDGFR- $\beta$  phosphorylation was observed when the cells were incubated with CM from the VEGF-C transfected cells, with or

without prior treatment with Flt4EC. In contrast, the addition of 50 ng/ml of PDGF-BB induced a prominent tyrosine autophosphorylation of PDGFR- $\beta$ .

#### EXAMPLE 15

##### VEGF-C Stimulates Endothelial Cell Migration In Collagen Gel

Conditioned media (CM) from cell cultures transfected with the VEGF-C expression vector was placed in a well made in collagen gel and used to stimulate the migration of bovine capillary endothelial (BCE) cells in the three-dimensional collagen gel as follows.

BCE cells (Folkman et al., *Proc. Natl. Acad. Sci. (USA)*, 76:5217-5221 (1979)) were cultured as described in Pertovaara et al., *J. Biol. Chem.*, 269:6271-74 (1994). The collagen gels were prepared by mixing type I collagen stock solution (5 mg/ml in 1 mM HCl) with an equal volume of 2xMEM and 2 volumes of MEM containing 10% newborn calf serum to give a final collagen concentration of 1.25 mg/ml. The tissue culture plates (5 cm diameter) were coated with about 1 mm thick layer of the solution, which was allowed to polymerize at 37°C. BCE cells were seeded on top of this layer. For the migration assays, the cells were allowed to attach inside a plastic ring (1 cm diameter) placed on top of the first collagen layer. After 30 minutes, the ring was removed and unattached cells were rinsed away. A second layer of collagen and a layer of growth medium (5% newborn calf serum (NCS)), solidified by 0.75% low melting point agar (FMC BioProducts, Rockland, Me.), were added. A well (3 mm diameter) was punched through all the layers on both sides of the cell spot at a distance of 4 mm, and the sample or control media were pipetted daily into the wells. Photomicrographs of the cells migrating out from the spot edge were taken after six days through an Olympus CK 2 inverted microscope equipped with phase-contrast optics. The migrating cells were counted after nuclear staining with the fluorescent dye bisbenzimidazole (1 mg/ml, Hoechst 33258, Sigma).

FIG. 4 depicts a comparison of the number of cells migrating at different distances from the original area of attachment towards wells containing media conditioned by the non-transfected (control) or transfected (mock; VEGF-C; VEGF) cells, 6 days after addition of the media. The number of cells migrating out from the original ring of attachment was counted in five adjacent 0.5 mm $\times$ 0.5 mm squares using a microscope ocular lens grid and 10x magnification with a fluorescence microscope. Cells migrating further than 0.5 mm were counted in a similar way by moving the grid in 0.5 mm steps. The experiments were carried out twice with similar results, and medium values from the one of the experiments are presented with standard error bars. As can be seen from the columns, VEGF-C-containing CM stimulated cell migration more than medium conditioned by the non-transfected or mock-transfected cells but less than medium from cells transfected with a VEGF expression vector. Daily addition of 1 ng of FGF2 into the wells resulted in the migration of approximately twice the number of cells when compared to the stimulation by CM from VEGF-transfected cells.

In related experiments, a "recombinantly-matured" VEGF-C polypeptide (VEGF-C  $\Delta$ NACHis, described below) was shown to stimulate the incorporation of  $^3$ H-thymidine into the DNA of BCE cells in a dose dependent manner (VEGF-C concentrations of 0, 10, 100, and 1000 pM tested). This data tends to confirm the observation,

immunization of rabbits at bi-weekly intervals using methods standard in the art (Harlow and Lane, *Antibodies, a laboratory manual*, Cold Spring Harbor Laboratory Press (1988)). Antisera obtained after the fourth booster immunization was used for immunoprecipitation of VEGF-C in pulse-chase experiments, as described below.

For pulse-chase analysis, 293 EBNA cells transfected with a VEGF-C expression vector (i.e., the FLT4-I. CDNA inserted into the pREP7 expression vector as described above) were incubated for 30 minutes in methionine-free, cysteine-free, serum-free DMEM culture medium at 37°C. The medium was then changed, and 200  $\mu$ Ci of Pro-mix<sup>TM</sup> (Amersham), was added. The cell layers were incubated in this labeling medium for two hours, washed with PBS, and incubated for 0, 15, 30, 60, 90, 120, or 180 minutes in serum-free DMEM (chase). After the various chase periods, the medium was collected, the cells were again washed two times in PBS, and lysed in immunoprecipitation buffer. The VEGF-C polypeptides were analyzed from both the culture medium and from the cell lysates by immunoprecipitation, using the VEGF-C-specific antiserum raised against the NH<sub>2</sub>-terminal peptide (PAM126) of the 23 kD VEGF-C form. Immunoprecipitated polypeptides were analyzed via SDS-PAGE followed by autoradiography.

The resultant autoradiograms demonstrated that immediately after a 2 hour labeling (chase time 0), the VEGF-C vector-transfected cells contained a radioactive polypeptide band of about 58 kD (originally estimated to be about 55 kD, and re-evaluated to be about 58 kD using different size standards), which was not observed in mock-transfected cells (M). Most of this ~58 kD precursor undergoes dimerization. This ~58 kD polypeptide band gradually diminished in intensity with increasing chase periods. A 32 kD polypeptide band also is observed in VEGF-C transfected cells (but not mock-transfected cells). This 32 kD band disappears from cells with similar kinetics to that of the ~58 kD band. Additional analysis indicated that the 32 kD band was a doublet of 29 kD and 31-32 kD forms, held together by disulfide bonds. Simultaneously, increasing amounts of 32 kD and subsequently 23 kD and 14-15 kD polypeptides appeared in the medium.

Collectively, the data from the pulse-chase experiments indicate that the ~58 kD intracellular polypeptide represents a pro-VEGF-C polypeptide, which is proteolytically cleaved either intracellularly or at the cell surface into the 29 kD and 31-32 kD polypeptides. The 29/31 kD form is secreted and simultaneously further processed by proteolysis into the 23 kD and 14-15 kD forms. In additional experiments, disulfide linked dimers of the 29 kD and 15 kD forms were observed. Without intending to be limited to a particular theory, it is believed that processing of the VEGF-C precursor occurs as removal of a signal sequence, removal of the COOH-terminal domain (BR3P), and removal of an amino terminal polypeptide, resulting in a VEGF-C polypeptide having the TEE... amino terminus.

At high resolution, the 23 kD polypeptide band appears as a closely-spaced polypeptide doublet, suggesting heterogeneity in cleavage or glycosylation.

#### EXAMPLE 20

##### Isolation of Mouse and Quail cDNA Clones Encoding VEGF-C

To clone a murine VEGF-C, approximately  $1 \times 10^6$  bacteriophage lambda clones of a commercially-available 12 day mouse embryonal cDNA library (lambda EX10x library, Novagen, catalog number 69632-1) were screened with a

radiolabeled fragment of human VEGF-C cDNA containing nucleotides 495 to 1661 of SEQ ID NO: 7. One positive clone was isolated.

A 1323 bp EcoRI/HindIII fragment of the insert of the isolated mouse cDNA clone was subcloned into the corresponding sites of the pBluescript SK+ vector (Stratagene) and sequenced. The cDNA sequence of this clone was homologous to the human VEGF-C sequence reported herein, except that about 710 bp of 5'-end sequence present in the human clone was not present in the mouse clone.

For further screening of mouse cDNA libraries, a HindIII-BstXI (HindIII site is from the pBluescript SK+ polylinker) fragment of 881 bp from the coding region of the mouse cDNA clone was radiolabeled and used as a probe to screen two additional mouse cDNA libraries. Two additional cDNA clones from an adult mouse heart ZAP II cDNA library (Stratagene, catalog number 936306) were identified. Three additional clones also were isolated from a mouse heart 5'-stretch-plus cDNA library in  $\lambda$ gt11 (Clontech Laboratories, Inc., catalog number ML5002b). Of the latter three clones, one was found to contain an insert of about 1.9 kb. The insert of this cDNA clone was subcloned into EcoRI sites of pBluescript SK+ vector and both strands of this clone were completely sequenced, resulting in the nucleotide and deduced amino acid sequences shown in SEQ ID NOs: 10 and 11.

It is contemplated that the polypeptide corresponding to SEQ ID NO: 11 is processed into a mature mouse VEGF-C protein, in a manner analogous to the processing of the human VEGF-C prepropeptide. Putative cleavage sites for the mouse protein are identified using procedures outlined above for identification of cleavage sites for the human VEGF-C polypeptide.

The foregoing results demonstrate the utility of polynucleotides of the invention for identifying and isolating polynucleotides encoding other non-human mammalian VEGF-C proteins. Such identified and isolated polynucleotides, in turn, can be expressed (using procedures similar to those described in preceding examples) to produce recombinant polypeptides corresponding to non-human mammalian forms of VEGF-C.

The mouse and human VEGF-C sequences were used to design probes for isolating a quail VEGF-C cDNA from a quail cDNA library. A fragment of the human VEGF-C cDNA comprising nucleotides 495-1670 of SEQ ID NO: 7 was obtained by PCR amplification, cloned into the pCRII vector (Invitrogen) according to the manufacturer's instructions, and amplified. The insert was isolated by EcoRI digestion and preparative gel electrophoresis and then labelled using radioactive dCTP and random priming. A cDNA library made from quail embryos of stage E-4 in pCDNA-1 vector (Invitrogen) was then screened using this probe. About 200,000 colonies were plated and filter replicas were hybridized with the radioactive probe. Nine positive clones were identified and secondarily plated. Two of the nine clones hybridized in secondary screening. The purified clones (clones 1 and 14) had approximately 2.7 kb EcoRI inserts. Both clones were amplified and then sequenced using the T7 and SP6 primers (annealing to the vector). In addition, an internal SphI restriction endonuclease cleavage site was identified about 1.9 kb from the T7 primer side of the vector and used for subcloning 5'- and 3'- SphI fragments, followed by sequencing from the SphI end of the subclones. The sequences obtained were identical from both clones and showed a high degree of similarity to the human VEGF-C coding region. Subsequently, walking primers



under light microscopy, that VEGF-C stimulates proliferation of these cells.

#### EXAMPLE 16

##### VEGF-C Is Expressed In Multiple Tissues

Northern blots containing 2 micrograms of isolated poly (A)<sup>+</sup> RNA from multiple human tissues (blot from Clontech Laboratories, Inc., Palo Alto, Calif.) were probed with radioactively labelled insert of the 2.1 kb VEGF-C cDNA clone. Northern blotting and hybridization analysis showed that the 2.4 kb RNA and smaller amounts of a 2.0 kb mRNA are expressed in multiple human tissues, most prominently in the heart, placenta, muscle, ovary and small intestine, and less prominently in prostate, colon, lung, pancreas, and spleen. Very little VEGF-C RNA was seen in the brain, liver, kidney, testis, or thymus and peripheral blood leukocytes (PBL) appeared negative. A similar analysis of RNA from human fetal brain, lung, liver, and kidney tissues showed that VEGF-C is highly expressed in the kidney and lung and to a lesser degree in the liver, while essentially no expression is detected in the brain. Interestingly, VEGF expression correlates with VEGF-C expression in these tissues, whereas VEGF-B is highly expressed in all four fetal tissues analyzed.

#### EXAMPLE 17

##### The VEGF-C Gene Localizes To Chromosome 4q34

A DNA panel of 24 interspecies somatic cell hybrids, which had retained one or two human chromosomes, was used for the chromosomal localization of the VEGF-C gene (Bios Laboratories, Inc., New Haven, Conn.). The procedures employed are described in detail in commonly-owned PCT patent application PCT/FI96/00427, filed Aug. 1, 1996, and commonly-owned U.S. patent application Ser. Nos. 08/671,573, filed Jun. 28, 1996; 08/601,132, filed Feb. 14, 1996; and 08/585,895, filed Jan. 12, 1996, all of which are incorporated by reference herein.

In order to determine the chromosomal localization of the human VEGF-C gene, DNAs from human rodent somatic cell hybrids containing defined sets of human chromosomes were analyzed by Southern blotting and hybridization with a VEGF-C cDNA probe. Among 24 DNA samples on the hybrid panel, representing different human chromosomes, human-specific signals were observed only in hybrids which contained human chromosome 4. The results were confirmed by PCR of somatic cell hybrid DNAs using VEGF-C specific primers, where amplified bands were obtained only from DNAs containing human chromosome 4.

A genomic P1 plasmid for VEGF-C was isolated using specific primers and PCR and verified by Southern blotting and hybridization using a VEGF-C specific cDNA probe. The chromosomal localization of VEGF-C was further studied using metaphase FISH. Using the P1 probe for VEGF-C in FISH a specific hybridization to the 4q34 chromosomal band was detected in 40 out of 44 metaphases. Double-fluorochrome hybridization using a cosmid probe specific for the aspartylglucosaminidase (AGA) gene showed that VEGF-C is located just proximal to the AGA gene previously mapped to the 4q34-35 chromosomal band.

Biotin labelled VEGF-C P1 and digoxigenin labeled AGA cosmid probes were hybridized simultaneously to metaphase chromosomes. This experiment demonstrated that the AGA gene is more telomerically located than the

VEGF-C gene. The foregoing example demonstrates the utility of polynucleotides of the invention as chromosomal markers and for the presence or absence of the VEGF-C gene region in normal or diseased cells. The VEGF-C locus at 4q34 is a candidate target for mutations leading to vascular malformations or cardiovascular diseases.

#### EXAMPLE 18

##### Effect of Glucose Concentration and Hypoxia on VEGF, VEGF-B and VEGF-C mRNA Levels in C6 Glioblastoma Cells

Confluent cultures of C6 cells (ATCC CCL 107) were grown on 10 cm diameter tissue culture plates containing 2.5 ml of DMEM and 5% fetal calf serum plus antibiotics. The cultures were exposed for 16 hours to normoxia in a normal cell culture incubator containing 5% CO<sub>2</sub> or hypoxia by closing the culture plates in an airtight glass chamber and burning a piece of wood inside until the flame was extinguished due to lack of oxygen. Polyadenylated RNA was isolated (as in the other examples), and 8 micrograms of the RNA was electrophoresed and blot-hybridized with a mixture of the VEGF, VEGF-B and VEGF-C probes. The results show that hypoxia strongly induces VEGF mRNA expression, both in low and high glucose, but has no significant effect on the VEGF-B mRNA levels. The VEGF-C mRNA isolated from hypoxic cells runs slightly faster in gel electrophoresis and an extra band of faster mobility can be seen below the upper mRNA band. This observation suggests that hypoxia affects VEGF-C RNA processing. One explanation for this observation is that VEGF-C mRNA splicing is altered, affecting the VEGF-C open reading frame and resulting in an alternative VEGF-C protein being produced by hypoxic cells. Such alternative forms of VEGF-C and VEGF-C-encoding polynucleotides are contemplated as an aspect of the invention. This data indicates screening and diagnostic utilities for polynucleotides and polypeptides of the invention, such as methods whereby a biological sample is screened for the hypoxia-induced form of VEGF-C and/or VEGF-C mRNA. The data further suggests a therapeutic indication for antibodies and/or other inhibitors of the hypoxia-induced form of VEGF-C or the normal form of VEGF-C.

#### EXAMPLE 19

##### Pulse-Chase Labeling and Immunoprecipitation of VEGF-C Polypeptides From 293 EBNA Cells Transfected With VEGF-C Expression Vector.

The following VEGF-C branched amino-terminal peptide, designated PAM126, was synthesized for production of anti-VEGF-C antiserum:

NH<sub>2</sub>-E-E-T-I-K-F-A-A-A-H-Y-N-T-E-I-L-K-COOH (SEQ ID NO: 9).

In particular, PAM126 was synthesized as a branched polylysine structure K3PA4 having four peptide acid (PA) chains attached to two available lysine (K) residues. The synthesis was performed on a 433A Peptide Synthesizer (Applied Biosystems) using Fmoc-chemistry and TentGel S MAP RAM10 resin mix (RAPP Polymere GmbH, Tubingen, Germany), yielding both cleavable and resin-bound peptides. The cleavable peptide was purified via reverse phase HPLC and was used together with the resin-bound peptide in immunizations. The correctness of the synthesis products were confirmed using mass-spectroscopy (Lasermat).

The PAM126 peptide was dissolved in phosphate buffered saline (PBS), mixed with Freund's adjuvant, and used for



were made in both directions and double-stranded sequencing was completed for 1743 base pairs, including the full-length open reading frame.

The cDNA sequence obtained includes a long open reading frame and 5' untranslated region. The DNA and deduced amino acid sequences for the quail cDNA are set forth in SEQ ID NOs: 12 and 13, respectively. Studies performed with the putative quail VEGF-C cDNA have shown that its protein product is secreted from transfected cells and interacts with avian VEGFR-3 and VEGFR-2, further confirming the conclusion that the cDNA encodes a quail VEGF-C protein. As shown in FIG. 5, the human, murine, and avian (quail) VEGF-C precursor amino acid sequences share a significant degree of conservation. This high degree of homology permits the isolation of VEGF-C encoding sequences from other species, especially vertebrate species, and more particularly mammalian and avian species, using polynucleotides of the present invention as probes and using standard molecular biological techniques such as those described herein.

#### EXAMPLE 21

##### N-terminal Peptide Sequence Analyses of Recombinant VEGF-C

Cells (293 EBNA) transfected with VEGF-C cDNA (see Example 13) secrete several forms of recombinant VEGF-C (FIG. 6A, lane IP). In the absence of alkylation, the three major, proteolytically-processed forms of VEGF-C migrate in SDS-PAGE as proteins with apparent molecular masses of 32/29 kD (doublet), 21 kD and 15 kD. Two minor polypeptides exhibit approximate molecular masses of 63 and 52 kD, respectively. More precise size measurements (using SDS-PAGE under reducing conditions) revealed that the molecular masses of the VEGF-C forms that were initially estimated as 63, 52, 32, 23, and 14 kD (using SDS-PAGE under reducing conditions and a different set of size standards) are approximately 58, 43, 31, 29, 21, and 15 kD, respectively (the initial measurements in most cases falling within acceptable 10% error of the more precise measurements). One of these polypeptides is presumably a glycosylated and non-processed form; the other polypeptide is presumably glycosylated and partially processed.

To determine sites of proteolytic cleavage of the VEGF-C precursor, an immunoaffinity column was used to purify VEGF-C polypeptides from the conditioned medium of 293 EBNA cells transfected with VEGF-C cDNA. To prepare the immunoaffinity column, a rabbit was immunized with a synthetic peptide corresponding to amino acids 104-120 of SEQ ID NO: 8:  $\text{H}_2\text{N}-\text{EETIKFAAAHYNTEILK}$  (see PAM126 in Example 19). The IgG fraction was isolated from the serum of the immunized rabbit using protein A Sepharose (Pharmacia). The isolated IgG fraction was covalently bound to CNBr-activated Sepharose CL-4B (Pharmacia) using standard techniques at a concentration of 5 mg IgG/ml of Sepharose. This immunoaffinity matrix was used to isolate processed VEGF-C from 1.2 liters of the conditioned medium (CM).

The purified material eluted from the column was analyzed by gel electrophoresis and Western blotting. Fractions containing VEGF-C polypeptides were combined, dialyzed against 10 mM Tris HCl, vacuum-dried, electrotransferred to Immobilon-P (polyvinylidene difluoride or PVDF) transfer membrane (Millipore, Marlborough, Mass.) and subjected to N-terminal amino acid sequence analysis.

The polypeptide band of 32 kD yielded two distinct sequences:  $\text{NH}_2\text{-FESGLDLSDA}$  . . . and  $\text{NH}_2\text{-}$

$\text{AVVMTQTPAS}$  . . . (SEQ ID NO: 14), the former corresponding to the N-terminal part of VEGF-C after cleavage of the signal peptide, starting from amino acid 32 (SEQ ID NO: 8), and the latter corresponding to the kappa-chain of IgG, which was present in the purified material due to "leakage" of the affinity matrix during the elution procedure.

In order to obtain the N-terminal peptide sequence of the 29 kD form of VEGF-C, a construct (VEGF-C NHis) encoding a VEGF-C mutant was generated. In particular, the construct encoded a VEGF-C mutant that fused a 6xHis tag to the N-terminus of the secreted precursor (i.e., between amino acids 31 and 33 in SEQ ID NO: 8). The phenylalanine at position 32 was removed to prevent possible cleavage of the tag sequence during secretion of VEGF-C. The VEGF-C NHis construct was cloned into pREP7 as a vector; the construction is described more fully in Example 28, below.

The calcium phosphate co-precipitation technique was used to transfect VEGF-C NHis into 293 EBNA cells. Cells were incubated in DMEM/10% fetal calf serum in 15 cm cell culture dishes (a total of 25 plates). The following day, the cells were reseeded into fresh culture dishes (75 plates) containing the same medium and incubated for 48 hours. Cell layers were then washed once with PBS and DMEM medium lacking FCS was added. Cells were incubated in this medium for 48 hours and the medium was collected, cleared by centrifugation at 5000xg and concentrated 500x using an Ultrasette Tangential Flow Device (Filtron, Northborough, Mass.), as described in Example 5 above. VEGF-C NHis was purified from the concentrated conditioned medium using TALON™ Metal Affinity Resin (Clontech Laboratories, Inc.) and the manufacturer's protocol for native protein purification using imidazole-containing buffers. The protein was eluted with a solution containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 200 mM imidazole. The eluted fractions containing purified VEGF-C NHis were detected by immunoblotting with Antiserum 882 (antiserum from rabbit 882, immunized with the PAM-126 polypeptide). Fractions containing VEGF-C NHis were combined, dialyzed and vacuum-dried. As can be seen in FIG. 11, due to the presence of the 6xHis tag at the N-terminus of this form of VEGF-C, the upper component of the major doublet of the VEGF-CNHis migrates slightly slower than the 32 kD form of wild type VEGF-C, thereby improving the separation of the VEGF-CNHis 32 kD mutant from the 29 kD band using SDS-PAGE. Approximately 15 µg of the purified VEGF-C were subjected to SDS-PAGE under reducing conditions, electrotransferred to Immobilon-P (PVDF) transfer membrane (Millipore, Inc., Marlborough, Mass.) and the band at 29 kD was subjected to N-terminal amino acid sequence analysis. This sequence analysis revealed an N-terminal sequence of  $\text{H}_2\text{N-SLPAT}$  . . . , corresponding to amino acids 228-232 of VEGF-C (SEQ ID NO: 8).

The polypeptide band of 21 kD yielded the sequence  $\text{H}_2\text{N-AHYNTEILKS}$  . . . , corresponding to an amino-terminus starting at amino acid 112 of SEQ ID NO: 8. Thus, the proteolytic processing site which results in the 21 kD form of VEGF-C produced by transfected 293 EBNA cells apparently occurs nine amino acid residues downstream of the cleavage site which results in the 23 kD form of VEGF-C secreted by PC-3 cells.

The N-terminus of the 15 kD form was identical to the N-terminus of the 32 kD form ( $\text{NH}_2\text{-FESGLDLSDA}$  . . . ). The 15 kD form was not detected when recombinant VEGF-C was produced by COS cells. This suggests that production of this form is cell lineage specific.

#### EXAMPLE 22

##### Dimeric and Monomeric Forms of VEGF-C

The composition of VEGF-C dimers was analyzed as follows. Cells (293 EBNA cells), transfected with the

pREP7 VEGF-C vector as described in Example 11, were metabolically labelled with Pro-mix L- [<sup>35</sup>S] labelling mix (Amersham Corp.) to a final concentration of 100  $\mu$ Ci/ml.

In parallel, a VEGF-C mutant, designated "R102S", was prepared and analyzed. To prepare the DNA encoding VEGF-C-R102S, the arginine codon at position 102 of SEQ ID NO: 8 was replaced with a serine codon. This VEGF-C-R102S-encoding DNA, in a pREP7 vector, was transfected into 293 EBNA cells and expressed as described above. VEGF-C polypeptides were immunoprecipitated using antisera 882 (obtained by immunization of a rabbit with a polypeptide corresponding to residues 104-120 of SEQ ID NO: 8 (see previous Example)) and antisera 905 (obtained by immunization of a rabbit with a polypeptide corresponding to a portion of the prepro- VEGF-C leader: H<sub>2</sub>N-ESGLDLSDAEPDAGEATAYASK (residues 33 to 54 of SEQ ID NO: 8).

The immunoprecipitates from each cell culture were subjected to SDS-PAGE under non-denaturing conditions (FIG. 6B). Bands 1-6 were cut out from the gel, soaked for 30 minutes in 1 $\times$ gel-loading buffer containing 200 mM  $\beta$ -mercaptoethanol, and individually subjected to SDS-PAGE under denaturing conditions (FIGS. 6A and 6C, lanes 1-6).

As can be seen from FIGS. 6A-C, each high molecular weight form of VEGF-C (FIG. 6B, bands 1-4) consists of at least two monomers bound by disulfide bonds (Compare FIGS. 6A and 6C, lanes 1-4, in the reducing gels). The main component of bands 1-3 is the doublet of 32/29 kD, where both proteins are present in an equimolar ratio. The main fraction of the 21 kD form is secreted as either a monomer or as a homodimer connected by means other than disulfide bonds (bands 6 and lanes 6 in FIGS. 6A-C).

The R102S mutation creates an additional site for N-linked glycosylation in VEGF-C at the asparagine residue at position 100 in SEQ ID NO: 8. Glycosylation at this additional glycosylation site increases the apparent molecular weight of polypeptides containing the site, as confirmed in FIGS. 6A-C and FIGS. 7A-B. The additional glycosylation lowers the mobility of forms of VEGF-C-R102S that contain the additional glycosylation site, when compared to polypeptides of similar primary structure corresponding to VEGF-C. FIGS. 6A-C and FIGS. 7A-B reveal that the VEGF-C-R102S polypeptides corresponding to the 32 kD and 15 kD forms of wt VEGF-C exhibit increased apparent molecular weights, indicating that each of these polypeptides contains the newly introduced glycosylation site. In particular, the VEGF-C-R102S polypeptide corresponding to the 15 kD polypeptide from VEGF-C comigrates on a gel with the 21 kD form of the wild type (wt) VEGF-C, reflecting a shift on the gel to a position corresponding to a greater apparent molecular weight. (Compare lanes 4 in FIGS. 6A and 6C). The mobility of the 58 kD form of VEGF-C was slowed to 64 kD by the R102S mutation, indicating that this form contains the appropriate N-terminal peptide of VEGF-C. The mobilities of the 21, 29, and 43 kD forms were unaffected by the R102S mutation, suggesting that these polypeptides contain peptide sequences located C-terminally of R<sub>102</sub>.

In a related experiment, another VEGF-C mutant, designated "R226,227S," was prepared and analyzed. To prepare a DNA encoding VEGF-C-R226,227S, the arginine codons at positions 226 and 227 of SEQ ID NO: 8 were replaced with serine codons by site-directed mutagenesis. The resultant DNA was transfected into 293 EBNA cells as described above and expressed and analyzed under the same condi-

tions as described for VEGF-C and VEGF-C-R102S. In the conditioned medium from the cells expressing VEGF-C-R226,227S, no 32 kD form of VEGF-C was detected. These results indicate that a C-terminal cleavage site of wild-type VEGF-C is adjacent to residues 226 and 227 of SEQ ID NO: 8, and is destroyed by the mutation of the arginines to serines. Again, the mobility of the 29 kD component of the doublet was unchanged (FIGS. 7A-B).

Taken together, these data indicate that the major form of the processed VEGF-C is a heterodimer consisting of (1) a polypeptide of 32 kD containing amino acids 32-227 of the prepro-VEGF-C (amino acids 32 to 227 in SEQ ID NO: 8) attached by disulfide bonds to (2) a polypeptide of 29 kD beginning with amino acid 228 in SEQ ID NO: 8. These data are also supported by a comparison of the pattern of immunoprecipitated, labelled VEGF-C forms using antisera 882 and antisera 905.

When VEGF-C immunoprecipitation was carried out using conditioned medium, both antisera (882 and 905) recognized some or all of the three major processed forms of VEGF-C (32/29 kD, 21 kD and 15 kD). When the conditioned medium was reduced by incubation in the presence of 10 mM dithiothreitol for two hours at room temperature with subsequent alkylation by additional incubation with 25 mM iodoacetamide for 20 minutes at room temperature, neither antibody precipitated the 29 kD component, although antibody 882 still recognized polypeptides of 32 kD, 21 kD and 15 kD. In subsequent experiments it was observed that neither antibody was capable of immunoprecipitating the 43 kD form. These results are consistent with the nature of the oligopeptide antigen used to elicit the antibodies contained in antisera 882, an oligopeptide containing amino acid residues 104-120 of SEQ ID NO: 8. On the other hand, antisera 905 recognized only the 32 kD and 15 kD polypeptides, which include sequence of the oligopeptide (amino acids 33 to 54 of SEQ ID NO: 8) used for immunization to obtain antisera 905. Taking into account the mobility shift of the 32 kD and 15 kD forms, the immunoprecipitation results with the R102S mutant were similar (FIGS. 8A-B). The specificity of antibody 905 is confirmed by the fact that it did not recognize a VEGF-C  $\Delta$ N form wherein the N-terminal propeptide spanning residues 32-102 of the unprocessed polypeptide had been deleted (FIG. 8B).

The results of these experiments also demonstrate that the 21 kD polypeptide is found (1) in heterodimers with other molecular forms (see FIGS. 6A-C and FIGS. 7A-B), and (2) secreted as a monomer or a homodimer held by bonds other than disulfide bonds (FIGS. 6A and 6B, lanes 6).

The experiments disclosed in this example demonstrate that several forms of VEGF-C exist. A variety of VEGF-C monomers were observed and these monomers can vary depending on the level and pattern of glycosylation. In addition, VEGF-C was observed as a multimer, for example a homodimer or a heterodimer. The processing of VEGF-C is schematically presented in FIG. 9 (disulfide bonds not shown). All forms of VEGF-C are within the scope of the present invention.

#### EXAMPLE 23

##### In Situ Hybridization of Mouse Embryos

To analyze VEGF-C mRNA distribution in different cells and tissues, sections of 12.5 and 14.5-day post-coitus (p.c.) mouse embryos were prepared and analyzed via in situ hybridization using labeled VEGF-C probes. In situ hybrid-

ization of tissue sections was performed as described in Vastrik et al., *J. Cell Biol.*, 128:1197-1208 (1995). A mouse VEGF-C antisense RNA probe was generated from linearized pBluescript II SK<sup>+</sup> plasmid (Stratagene Inc., La Jolla, Calif.), containing a CDNA fragment corresponding to nucleotides 499-979 of a mouse VEGF-C CDNA (SEQ ID NO: 10). Radiolabeled RNA was synthesized using T7 polymerase and [<sup>35</sup>S]-UTP (Amersham). Mouse VEGF-B antisense and sense RNA probes were synthesized in a similar manner from linearized PCR11 plasmid containing the mouse VEGF-B CDNA insert as described Olofsson et al., *Proc. Natl. Acad. Sci. (USA)*, 93:2576-2581 (1996). The high stringency wash was for 45 minutes at 65° C. in a solution containing 30 mM dithiothreitol (DTT) and 4xSSC. The slides were exposed for 28 days, developed and stained with hematoxylin. For comparison, similar sections were hybridized with a VEGFR-3 probe and the 12.5-day p.c. embryos were also probed for VEGF-B mRNA.

Darkfield and lightfield photomicrographs from these experiments are presented in commonly-owned PCT patent application PCT/FI96/00427, filed Aug. 01, 1996, incorporated by reference herein. Observations from the photomicrographs are summarized below. In a 12.5 day p.c. embryo, a parasagittal section revealed that VEGF-C mRNA was particularly prominent in the mesenchyme around the vessels surrounding the developing metanephros. In addition, hybridization signals were observed between the developing vertebrae, in the developing lung mesenchyme, in the neck region and developing forehead. The specificity of these signals was evident from the comparison with VEGF-B expression in an adjacent section, where the myocardium gave a very strong signal and lower levels of VEGF-B mRNA were detected in several other tissues. Both genes appear to be expressed in between the developing vertebrae, in the developing lung, and forehead. Hybridization of the VEGF-C sense probe showed no specific expression within these structures.

Studies also were conducted of the expression patterns of VEGF-C and VEGFR-3 in 12.5 day p.c. mouse embryos in the jugular region, where the developing dorsal aorta and cardinal vein are located. This is the area where the first lymphatic vessels sprout from venous sac-like structures according to the long-standing theory of Sabin, *Am. J. Anat.*, 9:43-91 (1909). An intense VEGF-C signal was detected in the mesenchyme surrounding the developing venous sacs which also were positive for VEGFR-3.

The mesenterium supplies the developing gut with blood and contains developing lymphatic vessels. The developing 14.5 day p.c. mesenterium is positive for VEGF-C mRNA, with particularly high expression in connective tissue surrounding certain vessels. The adjacent mesenterial VEGFR-3 signals that were observed originate from small capillaries of the mesenterium. Therefore, there appears to be a paracrine relationship between the production of the mRNAs for VEGF-C and its receptor. This data indicates that VEGF-C is expressed in a variety of tissues. Moreover, the pattern of expression is consistent with a role for VEGF-C in venous and lymphatic vessel development. Further, the data reveals that VEGF-C is expressed in non-human animals.

#### EXAMPLE 24

##### Analysis of VEGF, VEGF-B, and VEGF-C mRNA Expression in Fetal and Adult Tissues

A human fetal tissue Northern blot containing 2 µg of polyadenylated RNAs from brain, lung, liver and kidney

(Clontech Inc.) was hybridized with a pool of the following probes: a human full-length VEGF-C CDNA insert (Genbank Acc. No. X94216), a human VEGF-B<sub>167</sub> cDNA fragment (nucleotides 1-382, Genbank Acc. No. U48800) obtained by PCR amplification; and a human VEGF 581 bp cDNA fragment covering base pairs 57-638 (Genbank Acc. No. X15997). Blots were washed under stringent conditions, using techniques standard in the art.

Mouse embryo multiple tissue Northern blot (Clontech Inc.) containing 2 µg of polyadenylated RNAs from 7, 11, 15 and 17 day postcoital (p.c.) embryos was hybridized with mouse VEGF-C cDNA fragment (base pairs 499-656). A mouse adult tissue Northern blot was hybridized with the probes for human VEGF, VEGF-B<sub>167</sub>, VEGF-C and with a VEGFR-3 CDNA fragment (nucleotides 1-595; Genbank Acc. No. X68203).

In adult mouse tissues, both 2.4 kb and 2.0 kb mRNA signals were observed with the VEGF-C probe, at an approximately 4:1 ratio. The most conspicuous signals were obtained from lung and heart RNA, while kidney, liver, brain, and skeletal muscle had lower levels, and spleen and testis had barely visible levels. As in the human tissues, VEGF mRNA expression in adult mice was most abundant in lung and heart RNA, whereas the other samples showed less coordinate regulation with VEGF-C expression. Skeletal muscle and heart tissues gave the highest VEGF-B mRNA levels from adult mice, as previously reported Olofsson et al., *Proc. Natl. Acad. Sci. (USA)*, 93:2576-2581 (1996). Comparison with VEGFR-3 expression showed that the tissues where VEGF-C is expressed also contain mRNA for its cognate receptor tyrosine kinase, although in the adult liver VEGFR-3 mRNA was disproportionately abundant.

To provide a better insight into the regulation of the VEGF-C mRNA during embryonic development, polyadenylated RNA isolated from mouse embryos of various gestational ages (7, 11, 15, and 17 day p.c.) was hybridized with the mouse VEGF-C probe. These analyses showed that the amount of 2.4 kb VEGF-C mRNA is relatively constant throughout the gestational period.

#### EXAMPLE 25

##### Regulation of mRNAs for VEGF Family Members by Serum, Interleukin-1 and Dexamethasone in Human Fibroblasts in Culture

Human IMR-90 fibroblasts were grown in DMEM medium containing 10% FCS and antibiotics. The cells were grown to 80% confluence, then starved for 48 hours in 0.5% FCS in DMEM. Thereafter, the growth medium was changed to DMEM containing 5% FCS, with or without 10 ng/ml interleukin-1 (IL-1) and with or without 1 mM dexamethasone. The culture plates were incubated with these additions for the times indicated, and total cellular RNA was isolated using the TRIZOL kit (GIBCO-BRL). About 20 µg of total RNA from each sample was electrophoresed in 1.5% formaldehyde-agarose gels as described in Sambrook et al., *supra* (1989). The gel was used for Northern blotting and hybridization with radiolabeled insert DNA from the human VEGF clone (a 581 bp cDNA covering bps 57-638, Genbank Acc. No. 15997) and a human VEGF-B<sub>167</sub> cDNA fragment (nucleotides 1-382, Genbank Acc. No. U48800). Subsequently, the Northern blots were probed with radiolabelled insert from the VEGF-C cDNA plasmid. Primers were labelled using a standard technique involving enzymatic extension reactions of random primers, as would be understood by one of ordinary skill in the art.

The Northern blot analyses revealed that very low levels of VEGF-C and VEGF are expressed by the starved IMR-90 cells as well as cells after 1 hour of stimulation. In contrast, abundant VEGF-B mRNA signal was visible under these conditions. After 4 hours of serum stimulation, there was a strong induction of VEGF-C and VEGF mRNAs, which were further increased in the IL-1 treated sample. The effect of IL-1 seemed to be abolished in the presence of dexamethasone. A similar pattern of enhancement was observed in the 8 hour sample, but a gradual down-regulation of all signals was observed for both RNAs in the 24 hour and 48 hour samples. In contrast, VEGF-B mRNA levels remained constant and thus showed remarkable stability throughout the time period. The results are useful in guiding efforts to use VEGF-C and its fragments, its antagonists, and anti-VEGF-C antibodies in methods for treating a variety of disorders.

#### EXAMPLE 26

##### Expression and Analysis of Recombinant Murine VEGF-C

The mouse VEGF-C cDNA was expressed as a recombinant protein and the secreted protein was analyzed for its receptor binding properties. The binding of mouse VEGF-C to the human VEGFR-3 extracellular domain was studied by using media from Bosc23 cells transfected with mouse VEGF-C cDNA in a retroviral expression vector.

The 1.8 kb mouse VEGF-C cDNA was cloned as an EcoRI fragment into the retroviral expression vector pBabe-puro containing the SV40 early promoter region [Morgenstern et al., *Nucl. Acids Res.*, 18:3587-3595 (1990)], and transfected into the Bosc23 packaging cell line [Pearet et al., *Proc. Natl. Acad. Sci. (USA)*, 90:8392-8396 (1994)] by the calcium-phosphate precipitation method. For comparison, Bosc23 cells also were transfected with the previously-described human VEGF-C construct in the pREP7 expression vector. The transfected cells were cultured for 48 hours prior to metabolic labelling. Cells were changed into DMEM medium devoid of cysteine and methionine, and, after 45 minutes of preincubation and medium change, Pro-mix™ L- [<sup>35</sup>S] in vitro cell labelling mix (Amersham Corp.), in the same medium, was added to a final concentration of about 120  $\mu$ Ci/ml. After 6 hours of incubation, the culture medium was collected and clarified by centrifugation.

For immunoprecipitation, 1 ml aliquots of the media from metabolically-labelled Bosc23 cells transfected with empty vector or mouse or human recombinant VEGF-C, respectively, were incubated overnight on ice with 2  $\mu$ l of rabbit polyclonal antiserum raised against an N-terminal 17 amino acid oligopeptide of mature human VEGF-C (H<sub>2</sub>N-EETIKTAAAHYNTEILK) (SEQ ID NO: 8, residues 104-120). Thereafter, the samples were incubated with protein A sepharose for 40 minutes at 4° C. with gentle agitation. The sepharose beads were then washed twice with immunoprecipitation buffer and four times with 20 mM Tris-HCl, pH 7.4. Samples were boiled in Laemmli buffer and analyzed by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoprecipitation of VEGF-C from media of transfected and metabolically-labelled cells revealed bands of approximately 30-32 $\times$ 10<sup>3</sup> M<sub>r</sub> (a doublet) and 22-23 $\times$ 10<sup>3</sup> M<sub>r</sub> in 12.5% SDS-PAGE. These bands were not detected in samples from nontransfected or mock-transfected cells. These results show that antibodies raised against human VEGF-C recognize the corresponding mouse ligand.

For receptor binding experiments, 1 ml aliquots of media from metabolically-labelled Bosc23 cells were incubated with VEGFR-3 extracellular domain (see Example 3), covalently coupled to sepharose, for 4 hours at 4° C. with gentle mixing. The sepharose beads were washed four times with ice-cold phosphate buffered saline (PBS), and the samples were analyzed by gel electrophoresis as described in Joukov et al., *EMBO J.*, 15:290-298 (1996).

Similar 30-32 $\times$ 10<sup>3</sup> M<sub>r</sub> doublet and 22-23 $\times$ 10<sup>3</sup> M<sub>r</sub> polypeptide bands were obtained in the receptor binding assay as compared to the immunoprecipitation assay. Thus, mouse VEGF-C binds to human VEGFR-3. The slightly faster mobility of the mouse VEGF-C polypeptides that was observed may be caused by the four amino acid residue difference observed in sequence analysis (residues H88-E91, FIG. 10).

The capacity of mouse recombinant VEGF-C to induce VEGFR-3 autophosphorylation was also investigated. For the VEGFR-3 receptor stimulation experiments, subconfluent NIH 3T3-F14 cells, Pajusola et al., *Oncogene*, 9:3545-3555 (1994), were starved overnight in serum-free medium containing 0.2% BSA. In general, the cells were stimulated with the conditioned medium from VEGF-C vector-transfected cells for 5 minutes, washed three times with cold PBS containing 200  $\mu$ M vanadate, and lysed in RIPA buffer for immunoprecipitation analysis. The lysates were centrifuged for 25 minutes at 16000 $\times$ g and the resulting supernatants were incubated for 2 hours on ice with the specific antisera, followed by immunoprecipitation using protein A-sepharose and analysis in 7% SDS-PAGE. Polypeptides were transferred to nitrocellulose and analyzed by immunoblotting using anti-phosphotyrosine (Transduction Laboratories) and anti-receptor antibodies, as described by Pajusola et al., *Oncogene*, 9:3545-3555 (1994). Filter stripping was carried out at 50° C. for 30 minutes in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, with occasional agitation. The results of the experiment demonstrated that culture medium containing mouse VEGF-C stimulates the autophosphorylation of VEGFR-3 to a similar extent as human baculoviral VEGF-C or the tyrosyl phosphatase inhibitor pervanadate.

Mouse VEGF-C appeared to be a potent inducer of VEGFR-3 autophosphorylation, with the 195 $\times$ 10<sup>3</sup> M<sub>r</sub> precursor and proteolytically-cleaved 125 $\times$ 10<sup>3</sup> M<sub>r</sub> tyrosine kinase polypeptides of the receptor (Pajusola et al., *Oncogene*, 9:3545-3555 (1994)), being phosphorylated.

VEGFR-2 stimulation was studied in subconfluent porcine aortic endothelial (PAE) cells expressing Kdr (VEGFR-2) (PAE-VEGFR-2) [Waltenberger et al., *J. Biol. Chem.*, 269:26988-26995 (1994)], which were starved overnight in serum-free medium containing 0.2% BSA. Stimulation was carried out and the lysates prepared as described above. For receptor immunoprecipitation, specific antiserum for VEGFR-2 [Waltenberger et al., *J. Biol. Chem.*, 269:26988-26995 (1994)] was used. The immunoprecipitates were analyzed as described for VEGFR-3 in 7% SDS-PAGE followed by Western blotting with anti-phosphotyrosine antibodies, stripping of the filter, and re-probing it with anti-VEGFR-2 antibodies (Santa Cruz). VEGFR-2 stimulation was first tried with unconcentrated medium from cells expressing recombinant VEGF-C, but immunoblotting analysis did not reveal any receptor autophosphorylation.

To further determine whether mouse recombinant VEGF-C can also induce VEGFR-2 autophosphorylation as observed for human VEGF-C, PAE cells expressing

VEGFR-2 were stimulated with tenfold concentrated medium from cultures transfected with mouse VEGF-C expression vector and autophosphorylation was analyzed. For comparison, cells treated with tenfold concentrated medium containing human recombinant VEGF-C (Joukov et al., (1996)), unconcentrated medium from human VEGF-C baculovirus infected insect cells, or pervanadate (a tyrosyl phosphatase inhibitor) were used. In response to human baculoviral VEGF-C as well as pervanadate treatment, VEGFR-2 was prominently phosphorylated, whereas human and mouse recombinant VEGF-C gave a weak and barely detectable enhancement of autophosphorylation, respectively. Media from cell cultures transfected with empty vector or VEGF-C cloned in the antisense orientation did not induce autophosphorylation of VEGFR-2. Therefore, mouse VEGF-C binds to VEGFR-3 and activates this receptor at a much lower concentration than needed for the activation of VEGFR-2. Nevertheless, the invention comprehends methods for using the materials of the invention to take advantage of the interaction of VEGF-C with VEGFR-2, in addition to the interaction between VEGF-C and VEGFR-3.

#### EXAMPLE 27

##### VEGF-C E104-E213 Fragment Expressed in *Pichia* Yeast Stimulates Autophosphorylation of Flt4 (VEGFR-3) and KDR (VEGFR-2)

A truncated form of human VEGF-C cDNA was constructed wherein (1) the sequence encoding residues of a putative mature VEGF-C amino terminus H<sub>2</sub>N-E(104)ETIK (SEQ ID NO: 8, residues 104 et seq.) was fused in-frame to the yeast PHOI signal sequence (Invitrogen *Pichia* Expression Kit, Catalog #K1710-01), and (2) a stop codon was introduced after amino acid 213 (H<sub>2</sub>N-...RCMS; i.e., after codon 213 of SEQ ID NO: 7). The resultant truncated cDNA construct was then inserted into the *Pichia pastoris* expression vector pHL-S1 (Invitrogen). For the cloning, an internal BglII site in the VEGF-C coding sequence was mutated without change of the encoded polypeptide sequence.

This VEGF-C expression vector was then transfected into *Pichia* cells and positive clones were identified by screening for the expression of VEGF-C protein in the culture medium by Western blotting. One positive clone was grown in a 50 ml culture, and induced with methanol for various periods of time from 0 to 60 hours. About 10  $\mu$ l of medium was analyzed by gel electrophoresis, followed by Western blotting and detection with anti-VEGF-C antiserum, as described above. An approximately 24 kD polypeptide (band spreading was observed due to glycosylation) accumulated in the culture medium of cells transfected with the recombinant VEGF-C construct, but not in the medium of mock-transfected cells or cells transfected with the vector alone.

The medium containing the recombinant VEGF-C protein was concentrated by Centricon 30 kD cutoff ultrafiltration and used to stimulate NIH 3T3 cells expressing Flt4 (VEGFR-3) and porcine aortic endothelial (PAE) cells expressing KDR (VEGFR-2). The stimulated cells were lysed and immunoprecipitated using VEGFR-specific antisera and the immunoprecipitates were analyzed by Western blotting using anti-phosphotyrosine antibodies, chemiluminescence, and fluorography. As a positive control for maximal autophosphorylation of the VEGFRs, vanadate (VO<sub>4</sub>) treatment of the cells for 10 minutes was used. Medium from *Pichia* cultures secreting the recombinant VEGF-C polypeptide induced autophosphorylation of both Flt4 polypeptides of 195 kD and 125 kD as well as the

KDR polypeptide of about 200 kD. Vanadate, on the other hand, induces heavy tyrosyl phosphorylation of the receptor bands in addition to other bands probably coprecipitating with the receptors.

These results demonstrate that a VEGF-homologous domain of VEGF-C consisting of amino acid residues 104E-213S (SEQ ID NO: 8, residues 104-213) can be recombinantly produced in yeast and is capable of stimulating the autophosphorylation of Flt4 (VEGFR-3) and KDR (VEGFR-2). Recombinant VEGF-C fragments such as the fragment described herein, which are capable of stimulating Flt4 or KDR autophosphorylation are intended as aspects of the invention; methods of using these fragments are also within the scope of the invention.

#### EXAMPLE 28

##### Properties of the Differentially Processed Forms of VEGF-C

The following oligonucleotides were used to generate a set of VEGF-C variants and analogs:

5'-TCCTCTCTGCTGCTTGAAGTGGAG-3' (SEQ ID NO: 15), used to generate VEGF-C R102S (arginine mutated to serine at position 102 (SEQ ID NO: 8));

5'-TCCTCTCTGCTGCTTGAAGTGGAG-3' (SEQ ID NO: 16), used to generate VEGF-C R102G (arginine mutated to glycine at position 102 (SEQ ID NO: 8));

5'-TGTGCTGCAGCAAATTTATAGTCTCTCTCTGFGGCGGCGGGGCGCGGCGGCGCTCGCGAGGACC-3' (SEQ ID NO: 17), used to generate VEGF-C  $\Delta$ N (deletion of N-terminal propeptide corresponding to amino acids 32-102 (SEQ ID NO: 8));

5'-CTGGCAGGGAAGTCTAATAATGGAATGAA-3' (SEQ ID NO: 18), used to generate VEGF-C R226,227S (arginine codons mutated to serines at positions 226 and 227 (SEQ ID NO: 8));

5'-GGGCTCCGCTCCGAGAGGTGAGTCCGGA-CTCGTGATGGT GATGGTGATGGGCGGCGGCGGCGGCGGCGCTCGCGAGGACC-3' (SEQ ID NO: 19), used to generate VEGF-C NHis (this construct encodes a polypeptide with a 6xHis tag fused to approximately the N-terminus of the secreted precursor, as described in Example 21 (amino acid 33 of SEQ ID NO: 8)).

Some of the foregoing VEGF-C mutant constructs were further modified to obtain additional constructs. For example, VEGF-C R102G in pALTER (Promega) and oligonucleotide

5'-GTATTATAATGTCCTCCACCAAATTTTATAG-3' (SEQ ID NO: 20) were used to generate VEGF-C 4G, which encodes a polypeptide with four point mutations: R102G, A110G, A111G, and A112G (alanines mutated to glycines at positions 110-112 (SEQ ID NO: 8). These four mutations are adjacent to predicted sites of cleavage of VEGF-C expressed in PC-3 and recombinantly expressed in 293 EBNA cells.

Another construct was created using VEGF-C  $\Delta$ N and oligonucleotide

5'-GTTCTGCTGCCTGACACTGTGGTAGTGTGCTGGCGGCGGCTAGTGATGGTGATGGTGATGAATAATGGAATGAACCTGTCTGTAAACATCCAG-3' (SEQ ID NO: 21) to generate VEGF-C  $\Delta$ N $\Delta$ His. This construct encodes a polypeptide with a deleted N-terminal propeptide (amino acids 32-102); a deleted C-terminal propeptide (amino acids 226-419 of SEQ ID NO: 8); and an added 6xHis tag at the C-terminus.

All constructs were further digested with HindIII and NotI, subcloned into HindIII/NotI digested pREP7 vector,

and used to transfect 293 EBNA cells. About 48 hours after transfection, the cells were either metabolically labelled with Pro-mix<sup>TM</sup> as described above, or starved in serum-free medium for 2 days. Media were then collected and used in subsequent experiments. As can be seen from FIGS. 11A-B, wild type (wt) VEGF-C, VEGF-C NHis and VEGF-C  $\Delta$ NACHis were expressed to similar levels in 293 EBNA cells. At the same time, expression of the VEGF-C 4G polypeptide was considerably lower, possibly due to the changed conformation and decreased stability of the translated product. However, all the above VEGF-C mutants were secreted from the cells (compare FIGS. 11A and 11B).

The conditioned media from the transfected and starved cells were concentrated 5-fold and used to assess their ability to stimulate tyrosine phosphorylation of Flt4 (VEGFR-3) expressed in NIH 3T3 cells and KDR (VEGFR-2) expressed in PAE cells. Wild type (wt) VEGF-C, as well as all three mutant polypeptides, stimulated tyrosine phosphorylation of VEGFR-3. The most prominent stimulation observed was by the short mature VEGF-C  $\Delta$ NACHis. This mutant, as well as VEGF-C NHis, also stimulated tyrosine phosphorylation of VEGFR-2. Thus, despite the fact that a major component of secreted recombinant VEGF-C is a dimer of 32/29 kD, the active part of VEGF-C responsible for its binding to VEGFR-3 and VEGFR-2 is localized between amino acids 102 and 226 (SEQ ID NO: 8) of the VEGF-C precursor. Analysis and comparison of binding properties and biological activities of these VEGF-C proteins and mutants, using assays such as those described herein, will provide data concerning the significance of the observed major 32/29 kD and 21-23 kD VEGF-C processed forms. The data indicate that constructs encoding amino acid residues 103-225 of the VEGF-C precursor (SEQ ID NO: 8) generate a recombinant ligand that is functional for both VEGFR-3 and VEGFR-2.

The data from this and preceding examples demonstrate that numerous fragments of the VEGF-C polypeptide retain biological activity. A naturally occurring VEGF-C polypeptide spanning amino acids 103-226 (or 103-227) of SEQ ID NO: 8, produced by a natural processing cleavage defining the C-terminus, has been shown to be active. Example 27 demonstrates that a fragment with residues 104-213 of SEQ ID NO: 8 retains biological activity.

In addition, data from Example 21 demonstrates that a VEGF-C polypeptide having its amino terminus at position 112 of SEQ ID NO: 8 retains activity. Additional experiments have shown that a fragment lacking residues 1-112 of SEQ ID NO: 8 retains biological activity.

In a related experiment, a stop codon was substituted for the lysine at position 214 of SEQ ID NO: 8 (SEQ ID NO: 7, nucleotides 991-993). The resulting recombinant polypeptide still was capable of inducing Flt4 autophosphorylation, indicating that a polypeptide spanning amino acid residues 113-213 of SEQ ID NO: 8 is biologically active.

Sequence comparisons of members of the VEGF family of polypeptides provides an indication that still smaller fragments of the polypeptide depicted in SEQ ID NO: 8 will retain biological activity. In particular, eight highly conserved cysteine residues of the VEGF family of polypeptides define a region from residues 131-211 of SEQ ID NO: 8 (see FIG. 10) of evolutionary significance; therefore, a polypeptide spanning from about residue 131 to about residue 211 is expected to retain VEGF-C biological activity. In fact, a polypeptide which retains the conserved motif RCXXCC (e.g., a polypeptide comprising from about residue 161 to

about residue 211 of SEQ ID NO: 8 is postulated to retain VEGF-C biological activity. To maintain native conformation of these fragments, it may be preferred to retain about 1-2 additional amino acids at the carboxy-terminus and 1-2 or more amino acids at the amino terminus.

Beyond the preceding considerations, evidence exists that smaller fragments and/or fragment analogs which lack the conserved cysteines nonetheless will retain VEGF-C biological activity. Consequently, the materials and methods of the invention include all VEGF-C fragments, variants, and analogs that retain at least one biological activity of VEGF-C, regardless of the presence or absence of members of the conserved set of cysteine residues.

#### EXAMPLE 29

##### Expression of Human VEGF-C Under the Human K14 Keratin Promoter in Transgenic Mice Induces Abundant Growth of Lymphatic Vessels in the Skin

The Flt4 receptor tyrosine kinase is relatively specifically expressed in the endothelia of lymphatic vessels. Kaipainen et al., *Proc. Natl. Acad. Sci. (USA)*, 92: 3566-3570 (1995). Furthermore, the VEGF-C growth factor stimulates the Flt4 receptor, showing less activity towards the KDR receptor of blood vessels (Joukov et al., *EMBO J.*, 15: 290-298 (1996); See Example 26).

Experiments were conducted in transgenic mice to analyze the specific effects of VEGF-C overexpression in tissues. The human K14 keratin promoter is active in the basal cells of stratified squamous epithelia (Vassar et al., *Proc. Natl. Acad. Sci. (USA)*, 86:1563-1567 (1989)) and was used as the expression control element in the recombinant VEGF-C transgene. The vector containing the K14 keratin promoter is described in Vassar et al., *Genes Dev.*, 5:714-727 (1991) and Nelson et al., *J. Cell Biol.*, 97:244-251 (1983).

The recombinant VEGF-C transgene was constructed using the human full length VEGF-C cDNA (GenBank Acc. No. X94216). This sequence was excised from a pCI-neo vector (Promega) with XhoI/NotI, and the resulting 2027 base pair fragment containing the open reading frame and stop codon (nucleotides 352-1611 of SEQ ID NO: 7) was isolated. The isolated fragment was then subjected to an end-filling reaction using the Klenow fragment of DNA polymerase. The blunt-ended fragment was then ligated to a similarly opened BamHI restriction site in the K14 vector. The resulting construct contained the EcoRI site derived from the polylinker of the pCI-neo vector. This EcoRI site was removed using standard techniques (a Klenow-mediated fill-in reaction following partial digestion of the recombinant intermediate with EcoRI) to facilitate the subsequent excision of the DNA fragment to be injected into fertilized mouse oocytes.

The resulting clone, designated K14-VEGF-C, is illustrated in FIG. 20 of commonly-owned PCT patent application PCT/FI96/00427, filed Aug. 01, 1996.

The EcoRI-HindIII fragment from clone K14 VEGF-C containing the K14 promoter, VEGF-C cDNA, and K14 polyadenylation signal was isolated and injected into fertilized oocytes of the FVB-NIH mouse strain. The injected zygotes were transplanted to oviducts of pseudopregnant C57BL/6xDBA/2J hybrid mice. The resulting founder mice were analyzed for the presence of the transgene by polymerase chain reaction of tail DNA using the primers: 5'-CATGTACGAACCGCCAG-3' (SEQ ID NO: 22) and 5'-AATGACCAGAGAGAGGCGAG-3' (SEQ ID NO: 23).

In addition, the tail DNAs were subjected to EcoRV digestion and subsequent Southern analysis using the EcoRI-HindIII fragment injected into the mice. Out of 8 pups analyzed at 3 weeks of age, 2 were positive, having approximately 40–50 copies and 4–6 copies of the transgene in their respective genomes.

The mouse with the high copy number transgene was small, developed more slowly than its litter mates and had difficulty eating (i.e., suckling). Further examination showed a swollen, red snout and poor fur. Although fed with a special liquid diet, it suffered from edema of the upper respiratory and digestive tracts after feeding and had breathing difficulties. This mouse died eight weeks after birth and was immediately processed for histology, immunohistochemistry, and in situ hybridization.

Histological examination showed that in comparison to the skin of littermates, the dorsal dermis of K14-VEGF-C transgenic mice was atrophic and connective tissue was replaced by large lacunae devoid of red cells, but lined with a thin endothelial layer. These distended vessel-like structures resembled those seen in human lymphangiomas. The number of skin adnexal organs and hair follicles were reduced. In the snout region, an increased number of vessels was also seen. Therefore, VEGF-C overexpression in the basal epidermis is capable of promoting the growth of extensive vessel structure in the underlying skin, including large vessel lacunae. The endothelial cells surrounding these lacunae contained abundant Flt4 mRNA in in situ hybridization (see Examples 23 and 30 for methodology). The vessel morphology indicates that VEGF-C stimulates the growth of vessels having features of lymphatic vessels. The other K14-VEGF-C transgenic mouse had a similar skin histopathology.

Nineteen additional pups were analyzed at 3 weeks of age for the presence of the VEGF-C transgene, bring the number of analyzed pups to twenty-seven. A third transgene-positive pup was identified, having approximately 20 copies of the transgene in its genome. The 20 copy mouse and the 4–6 copy mouse described above transmitted the gene to 6 out of 11 and 2 out of 40 pups, respectively. The physiology of these additional transgenic mice were further analyzed.

The adult transgenic mice were small and had slightly swollen eyelids and poorly developed fur. Histological examination showed that the epidermis was hyperplastic and the number of hair follicles was reduced; these effects were considered unspecific or secondary to other phenotypic changes. The dermis was atrophic (45% of the dermal thickness, compared to 65% in littermate controls) and its connective tissue was replaced by large dilated vessels devoid of red cells, but lined with a thin endothelial cell layer. Such abnormal vessels were confined to the dermis and resembled the dysfunctional, dilated spaces characteristic of hyperplastic lymphatic vessels. See Fossum, et al., *J. Vet. Int. Med.*, 6: 283–293 (1992). Also, the ultrastructural features were reminiscent of lymphatic vessels, which differ from blood vessels by having overlapping endothelial junctions, anchoring filaments in the vessel wall, and a discontinuous or even partially absent basement membrane. See Leak, *Microvasc. Res.*, 2: 361–391 (1970). Furthermore, antibodies against collagen types IV, XVIII [Muragaki et al., *Proc. Natl. Acad. Sci. USA*, 92: 8763–8776 (1995)] and laminin gave very weak or no staining of the vessels, while the basement membrane staining of other vessels was prominent. The endothelium was also characterized by positive staining with monoclonal antibodies against desmoplakins I and II (Progen), expressed in lymphatic, but not in vascular endothelial cells. See Schmelz et al., *Differentiation*, 57:

97–117 (1994). Collectively, these findings strongly suggested that the abnormal vessels were of lymphatic origin.

In Northern hybridization studies, abundant VEGF-C mRNA was detected in the epidermis and hair follicles of the transgenic mice, while mRNAs encoding its receptors VEGFR-3 and VEGFR-2 as well as the Tie-1 endothelial receptor tyrosine kinase [Korhonen et al., *Oncogene*, 9: 395–403 (1994)] were expressed in endothelial cells lining the abnormal vessels. In the skin of littermate control animals, VEGFR-3 could be detected only in the superficial subpapillary layer of lymphatic vessels, while VEGFR-2 was found in all endothelia, in agreement with earlier findings. See Millauer et al., *Cell*, 72: 1–20 (1993); and Kaipainen et al., *Proc. Natl. Acad. Sci. USA*, 92: 3566–3570 (1995).

The lymphatic endothelium has a great capacity to distend in order to adapt to its functional demands. To determine whether vessel dilation was due to endothelial distension or proliferation, in vitro proliferation assays were conducted. Specifically, to measure DNA synthesis, 3mm×3mm skin biopsies from four transgenic and four control mice were incubated in D-MEM with 10 micrograms/ml BrdU for 6 hours at 37° C., fixed in 70% ethanol for 12 hours, and embedded in paraffin. After a 30 minute treatment with 0.1% pepsin in 0.1 M HCl at room temperature to denature DNA, staining was performed using mouse monoclonal anti-BrdU antibodies (Amersham). It appeared that the VEGF-C-receptor interaction in the transgenic mice transduced a mitogenic signal, because, in contrast to littermate controls, the lymphatic endothelium of the skin from young K14-VEGF-C mice showed increased DNA synthesis as demonstrated by BrdU incorporation followed by staining with anti-BrdU antibodies. This data further confirms that VEGF-C acts as a true growth factor in mammalian tissues.

In related experiments, a similar VEGF transgene did not induce lymphatic proliferation, but caused enhanced density of hyperpermeable, tortuous blood microvessels instead.

Angiogenesis is a multistep process which includes endothelial proliferation, sprouting, and migration. See Folkman et al., *J. Biol. Chem.*, 267: 10931–10934 (1992). To estimate the contribution of such processes to the transgenic phenotype, the morphology and function of the lymphatic vessels was analysed using fluorescent microlymphography using techniques known in the art. See Leu et al., *Am. J. Physiol.*, 267: 1507–1513 (1994); and Swartz et al., *Am. J. Physiol.*, 270: 324–329 (1996). Briefly, eight-week old mice were anesthetized and placed on a heating pad to maintain a 37° C. temperature. A 30-gauge needle, connected to a catheter filled with a solution of FITC-dextran 2M (8 mg/ml in PBS), was injected intradermally into the tip of the tail. The solution was infused with a constant pressure of 50 cm water (averaging roughly 0.01 microliters per minute flow rate) until the extent of network filling remained constant (approximately 2 hours). Flow rate and fluorescence intensity were monitored continuously throughout the experiment. In these experiments, a typical honeycomb-like network with similar mesh sizes was observed in both control and transgenic mice, but the diameter of lymphatic vessels was about twice as large in the transgenic mice, as summarized in the table below. (The intravital fluorescence microscopy of blood vessels was performed as has been described in the art. See Fukumura et al., *Cancer Res.*, 55: 4824–4829 (1995).)



Structural parameters of lymphatic and blood vessel networks

		transgenic	control	P-value**
lymphatic vessels*		(n = 4)	(n = 5)	
	diameter	142.3 ± 26.2	58.2 ± 21.7	.0143
	horizontal mesh size***	1003 ± 87.1	950.8 ± 93.1	.2207
	vertical mesh size	507.3 ± 58.9	488.8 ± 59.9	.5403
blood vessels		(n = 3)	(n = 6)	
	median diameter	8.3 ± 0.6	7.6 ± 1.1	.1213
	vessel density, cm/cm	159.2 ± 6.6	216.4 ± 20.0	.3017

n = number of animals

\*mean (μm) ± SD

\*\*Mann-Whitney test

\*\*\*mesh size describes vessel density

Some dysfunction of the abnormal vessels was indicated by the fact that it took longer for the dextran to completely fill the abnormal vessels. Injection of FITC-dextran into the tail vein, followed by fluorescence microscopy of the ear, showed that the blood vascular morphology was unaltered and leukocyte rolling and adherence appeared normal in the transgenic mice. These results suggest that the endothelial proliferation induced by VEGF-C leads to hyperplasia of the superficial lymphatic network but does not induce the sprouting of new vessels.

These effects of VEGF-C overexpression are unexpectedly specific, especially since, as described in other examples, VEGF-C is also capable of binding to and activating VEGFR-2, which is the major mitogenic receptor of blood vessel endothelial cells. In culture, high concentrations of VEGF-C stimulate the growth and migration of bovine capillary endothelial cells which express VEGFR-2, but not significant amounts of VEGFR-3. In addition, VEGF-C induces vascular permeability in the Miles assay [Miles, A. A., and Miles, E. M., *J. Physiol.*, 118:228-257 (1952); and Udaka, et al., *Proc. Soc. Exp. Biol. Med.*, 133:1384-1387 (1970)], presumably via its effect on VEGFR-2. VEGF-C is less potent than VEGF in the Miles assay, 4- to 5-fold higher concentrations of VEGF-C ΔNΔCHis being required to induce the same degree of permeability. In vivo, the specific effects of VEGF-C on lymphatic endothelial cells may reflect a requirement for the formation of VEGFR-3xVEGFR-2 heterodimers for endothelial cell proliferation at physiological concentrations of the growth factor. Such possible heterodimers may help to explain how three homologous VEGFs exert partially redundant, yet strikingly specific biological effects.

The foregoing in vivo data indicates utilities for both (i) VEGF-C polypeptides and polypeptide variants and analogs having VEGF-C biological activity, and (ii) anti-VEGF-C antibodies and VEGF-C antagonists that inhibit VEGF-C activity (e.g., by binding VEGF-C or interfering with VEGF-C/receptor interactions. For example, the data indicates a therapeutic utility for VEGF-C polypeptides in patients wherein growth of lymphatic tissue may be desirable (e.g., in patients following breast cancer or other surgery where lymphatic tissue has been removed and where lymphatic drainage has therefore been compromised, resulting in swelling; or in patients suffering from elephantiasis). The data indicates a therapeutic utility for anti-VEGF-C antibody substances and VEGF-C antagonists for conditions wherein growth-inhibition of lymphatic tissue may be desirable (e.g., treatment of lymphangiomas). Accordingly, meth-

ods of administering VEGF-C and VEGF-C variants, analogs, and antagonists are contemplated as methods and materials of the invention.

## EXAMPLE 30

## Expression of VEGF-C and Flt4 in the Developing Mouse

Embryos from a 16-day post-coitus pregnant mouse were prepared and fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and sectioned at 6 μm. The sections were placed on silanated microscope slides and treated with xylene, rehydrated, fixed for 20 minutes in 4% PFA, treated with proteinase K (7 mg/ml; Merck, Darmstadt, Germany) for 5 minutes at room temperature, again fixed in 4% PFA and treated with acetic anhydride, dehydrated in solutions with increasing ethanol concentrations, dried and used for in situ hybridization.

In situ hybridization of sections was performed as described (Vastrik et al., *J. Cell Biol.*, 128:1197-1208 (1995)). A mouse VEGF-C antisense RNA probe was generated from linearized pBluescript II SK+ plasmid (Stratagene Inc.), containing a fragment corresponding to nucleotides 499-979 of mouse VEGF-C cDNA, where the noncoding region and the BR3P repeat were removed by Exonuclease III treatment. The fragment had been cloned into the EcoRI and HindIII sites of pBluescript II SK+. Radiolabeled RNA was synthesized using T7 RNA Polymerase and [<sup>35</sup>S]-UTP (Amersham, Little Chalfont, UK). About two million cpm of the VEGF-C probe was applied per slide. After an overnight hybridization, the slides were washed first in 2xSSC and 20-30 mM DDT for 1 hour at 50° C. Treatment continued with a high stringency wash, 4xSSC and 20 mM DTT and 50% deionized formamide for 30 minutes at 65° C. followed by RNase A treatment (20 μg/ml) for 30 minutes at 37° C. The high stringency wash was repeated for 45 minutes. Finally, the slides were dehydrated and dried for 30 minutes at room temperature. The slides were dipped into photography emulsion and exposed for 4 weeks. Slides were developed using Kodak D-16 developer, counterstained with hematoxylin and mounted with Permount (FisherChemical).

For in situ hybridizations of Flt4 sequences, a mouse Flt4 CDNA fragment covering bp 1-192 of the published sequence (Finnerty et al., *Oncogene*, 8:2293-2298 (1993)) was used, and the above-described protocol was followed, with the following exceptions. Approximately one million cpm of the Flt4 probe were applied to each slide. The stringent washes following hybridization were performed in 1xSSC and 30 mM DTT for 105 minutes.

Darkfield and lightfield photomicrographs from these experiments are presented in commonly-owned PCT patent application PCT/F196/00427, filed Aug. 1, 1996, incorporated by reference herein. Observations from the photomicrographs are summarized below.

The most prominently Flt4-hybridizing structures appeared to correspond to the developing lymphatic and venous endothelium. A plexus-like endothelial vascular structure surrounding the developing nasopharyngeal mucous membrane was observed. The most prominent signal using the VEGF-C probe was obtained from the posterior part of the developing nasal conchae, which in higher magnification showed the epithelium surrounding loose connective tissue/forming cartilage. This structure gave a strong in situ hybridization signal for VEGF-C. With the VEGF-C probe, more weakly hybridizing areas were observed around



the snout, although this signal is much more homogeneous in appearance. Thus, the expression of VEGF-C is strikingly high in the developing nasal conchae.

The conchae are surrounded with a rich vascular plexus, important in nasal physiology as a source for the mucus produced by the epithelial cells and for warming inhaled air. It is suggested that VEGF-C is important in the formation of the conchal venous plexus at the mucous membranes, and that it may also regulate the permeability of the vessels needed for the secretion of nasal mucus. Possibly, VEGF-C and its derivatives, and antagonists, could be used in the regulation of the turgor of the conchal tissue and mucous membranes and therefore the diameter of the upper respiratory tract, as well as the quantity and quality of mucus produced. These factors are of great clinical significance in inflammatory (including allergic) and infectious diseases of the upper respiratory tract. Accordingly, the invention contemplates the use of the materials of the invention, including VEGF-C, Flt4, and their derivatives, in methods of diagnosing and treating inflammatory and infectious conditions affecting the upper respiratory tract, including nasal structures.

#### EXAMPLE 31

##### Characterization of the Exon-intron Organization of the Human VEGF-C Gene

Two genomic DNA clones covering exons 1, 2, and 3 of the human VEGF-C gene were isolated from a human genomic DNA library using VEGF-C cDNA fragments as probes. In particular, a human genomic library in bacteriophage EMBL-3 lambda (Clontech) was screened using a PCR-generated fragment corresponding to nucleotides 629-746 of the human VEGF-C cDNA (SEQ ID NO: 7). One positive clone, designated "lambda 3," was identified, and the insert was subcloned as a 14 kb XhoI fragment into the pBluescript II (pBSK II) vector (Stratagene). The genomic library also was screened with a labeled 130 bp NotI-SacI fragment from the 5'-noncoding region of the VEGF-C cDNA (the NotI site is in the polylinker of the cloning vector; the SacI site corresponds to nucleotides 92-97 of SEQ ID NO: 7). Two positive clones, designated "lambda 5" and "lambda 8," were obtained. Restriction mapping analysis showed that clone lambda 3 contains exons 2 and 3, while clone lambda 5 contains exon 1 and the putative promoter region.

Three genomic fragments containing exons 4, 5, 6 and 7 were subcloned from a genomic VEGF-C P1 plasmid clone. In particular, purified DNA from a genomic P1 plasmid clone 7660 (Paavonen et al., *Circulation*, 93: 1079-1082 (1996)) was used. EcoRI fragments of the P1 insert DNA were ligated into pBSK II vector. Subclones of clone 7660 which contained human VEGF-C cDNA homologous sequences were identified by colony hybridization, using the full-length VEGF-C cDNA as a probe. Three different genomic fragments were identified and isolated, which contained the remaining exons 4-7.

To determine the genomic organization, the clones were mapped using restriction endonuclease cleavage. Also, the coding regions and exon-intron junctions were partially sequenced. The result of this analysis is depicted in FIGS. 12 and 13A. The sequences of all intron-exon boundaries (FIG. 13A, SEQ ID NOs: 24-35) conformed to the consensus splicing signals (Mount, *Nucl. Acids Res.*, 10: 459-472 (1982)). The length of the intron between exon 5 and 6 was determined directly by nucleotide sequencing and found to

be 301 bp. The length of the intron between exons 2 and 3 was determined by restriction mapping and Southern hybridization and was found to be about 1.6 kb. Each of the other intron is over 10 kb in length.

A similar analysis was performed for the murine genomic VEGF-C gene. The sequences of murine VEGF-C intron-exon boundaries are depicted in FIG. 13B and SEQ ID NOs: 36-47.

The restriction mapping and sequencing data indicated that the VEGF-C signal sequence and the first residues of the N-terminal propeptide are encoded by exon 1. The second exon encodes the carboxy-terminal portion of the N-terminal propeptide and the amino terminus of the VEGF homology domain. The most conserved sequences of the VEGF homology domain are distributed in exons 3 (containing 6 conserved cysteine residues) and 4 (containing 2 cys residues). The remaining exons encode cysteine-rich motifs of the type C-6X-C-10X-CRC (exons 5 and 7) and a fivefold repeated motif of type C-6X-B-3X-C-C-C, which is typical of a silk protein.

To further characterize the human VEGF-C gene promoter, the lambda 5 clone was further analyzed. Restriction mapping of this clone using a combination of single- and double-digestions and Southern hybridizations indicated that it includes: (1) an approximately 6 kb region upstream of the putative initiator ATG codon, (2) exon 1, and (3) at least 5 kb of intron 1 of the VEGF-C gene.

A 3.7 kb Xba I fragment of clone lambda 5, containing exon 1 and 5' and 3' flanking sequences, was subcloned and further analyzed. As reported previously, a major VEGF-C mRNA band migrates at a position of about 2.4 kb. Calculating from the VEGF-C coding sequence of 1257 bp and a 391 bp 3' noncoding sequence plus a polyA sequence of about 50-200 bp, the mRNA start site was estimated to be about 550-700 bp upstream of the translation initiation codon.

RNAse protection assays were employed to obtain a more precise localization of the mRNA start site. The results of these experiments indicated that the RNA start site in the human VEGF-C gene is located 539 bp upstream of the ATG translational initiation codon.

To further characterize the promoter of the human VEGF-C gene, a genomic clone encompassing about 2.4 kb upstream of the translation initiation site was isolated, and the 5' noncoding cDNA sequence and putative promoter region were sequenced. The sequence obtained is set forth in SEQ ID NO: 48. (The beginning of the VEGF-C cDNA sequence set forth in SEQ ID NO: 7 corresponds to position 2632 of SEQ ID NO: 48; the translation initiation codon corresponds to positions 2983-2985 of SEQ ID NO: 48.) Similar to what has been observed with the VEGF gene, the VEGF-C promoter is rich in G and C residues and lacks consensus TATA and CCAAT sequences. Instead, it has numerous putative binding sites (5'-GGGCGG-3' or 5'-CCGCCC-3') for Sp1, a ubiquitous nuclear protein that can initiate transcription of TATA-less genes. See Pugh and Tjian, *Genes and Dev.*, 5:105-119 (1991). In addition, sequences upstream of the VEGF-C translation start site were found to contain frequent consensus binding sites for the AP-2 factor (5'-GCCN<sub>3</sub>GCC-3') and binding sites for the AP-1 factor (5'-TKASTCA-3'). Binding sites for regulators of tissue-specific gene expression, like NFkB and GATA, are located in the distant part of VEGF-C promoter. This suggests that the cAMP-dependent protein kinase and protein kinase C, as activators of AP-2 transcription factor [Curran and Franza, *Cell*, 55:395-397 (1988)], mediate VEGF-C transcriptional regulation.

The VEGF-C gene is abundantly expressed in adult human tissues, such as heart, placenta, ovary and small intestine, and is induced by a variety of factors. Indeed, several potential binding sites for regulators of tissue-specific gene expression, like NFkB (5'-GGGRNTY'YC-3') and GATA, are located in the distal part of the VEGF-C promoter. For example, NFkB is known to regulate the expression of tissue factor in endothelial cells. Also, transcription factors of the GATA family are thought to regulate cell-type specific gene expression.

Unlike VEGF, the VEGF-C gene does not contain a binding site for the hypoxia-inducible factor, HIF-1 (Levy et al., *J. Biol. Chem.*, 270: 13333-13340 (1995)). This finding suggests that if the VEGF-C mRNA is regulated by hypoxia, the mechanism would be based mainly on the regulation of mRNA stability. In this regard, numerous studies have shown that the major control point for the hypoxic induction of the VEGF gene is the regulation of the steady-state level of mRNA. See Levy et al., *J. Biol. Chem.*, 271: 2746-2753 (1996). The relative rate of VEGF mRNA stability and decay is considered to be determined by the presence of specific sequence motifs in its 3' untranslated region (UTR), which have been demonstrated to regulate mRNA stability. (Chen and Shyu, *Mol. Cell Biol.*, 14: 8471-8482 (1994)). The 3'-UTR of the VEGF-C gene also contains a putative motif of this type (TTATTT), at positions 1873-1878 of SEQ ID NO: 7.

To identify DNA elements important for basal expression of VEGF-C in transfected cells, a set of luciferase reporter plasmids containing serial 5' deletions through the promoter region was constructed. Restriction fragments of genomic DNA containing 5' portions of the first exon were cloned into the polylinker of the pGL3 reporter vector (Promega) and confirmed by sequencing. About 10 µg of the individual constructs in combination with 2 µg of pSV2-β-galactosidase plasmid (used as a control of transfection efficiency) were transfected into HeLa cells using the calcium phosphate-mediated transfection method. Two days after transfection, the cells were harvested and subjected to the luciferase assay. The luciferase activity was normalized to that of the pGL3 control vector driven by SV40 promoter/enhancer.

As depicted in FIG. 15, the 5.5 kb XbaI-RsrII fragment of clone lambda 5 gave nearly 9-fold elevated activity when compared with a promoterless vector. Deletion of a 5' XbaI-HindIII fragment of 2 kb had no effect on the promoter activity. The activity of the 1.16 kb XbaI-RsrII fragment was about twice that of the pGL3 basic vector, while the activity of the same fragment in the reverse orientation was at background level. Further deletion of the XbaI-SacI fragment caused an increase in the promoter activity, suggesting the presence of silencer elements in the region from -1057 to -199 (i.e., 199 to 1057 bp upstream from the transcription initiation site). The shortest fragment (SacII-RsrII) yielded only background activity, which was consistent with the fact that the mRNA initiation site was not present in this construct.

To determine whether further sequences in the first exon of human VEGF-C are important for basal expression, an RsrII fragment spanning nucleotides 214-495 (i.e., 214-495 bp downstream from the transcription initiation site) was subcloned in between of XbaI-RsrII fragment and the luciferase reporter gene. Indeed, the obtained construct showed an 50 % increase in activity when compared with the XbaI-RsrII-construct.

The VEGF gene has been shown to be up-regulated by a number of stimuli including serum derived growth factors.

To find out whether the VEGF-C gene also can be stimulated by serum, RNA from serum-starved and serum-stimulated HT1080 cells was subjected to primer extension analysis, which demonstrated that VEGF-C mRNA is up-regulated by serum stimulation.

Additional serum stimulation experiments indicated that the serum stimulation leads to increased VEGF-C promoter activity. Cells were transfected as described above and 24 h after transfection changed into medium containing 0.5% bovine serum albumin. Cells were then stimulated with 10% fetal calf serum for 4 hours and analyzed. The XbaI-RsrII promoter construct derived from lambda 5 yielded a twofold increased activity upon serum stimulation, while the same fragment in the reverse orientation showed no response. All other promoter constructs also showed up-regulation, ranging from 1.4 to 1.6 fold (FIG. 15).

#### EXAMPLE 32

##### Identification of a VEGF-C Splice Variant

As reported in Example 16, a major 2.4 kb VEGF-C mRNA and smaller amounts of a 2.0 kb mRNA are observable. To clarify the origin of these RNAs, several additional VEGF-C cDNAs were isolated and characterized. A human fibrosarcoma cDNA library from HT1080 cells in the lambda gt11 vector (Clontech, product #HL1048b) was screened using a 153 bp human VEGF-C cDNA fragment as a probe as described in Example 10. See also Joukov et al., *EMBO J.*, 15:290-298 (1996). Nine positive clones were picked and analyzed by PCR amplification using oligonucleotides 5'-CACGGCTTATGCAAGCAAAG-3' (SEQ ID NO: 49) and 5'-AACACAGTTTCCATAATAG-3' (SEQ ID NO: 50). These oligonucleotides were selected to amplify the portion of the VEGF-C cDNA corresponding to nucleotides 495-1661 of SEQ ID NO: 7. PCR was performed using an annealing temperature of 55° C. and 25 cycles.

The resultant PCR products were electrophoresed on agarose gels. Five clones out of the nine analyzed generated PCR fragments of the expected length of 1147 base pairs, whereas one was slightly shorter. The shorter fragment and one of the fragments of expected length were cloned into the pCRTMII vector (Invitrogen) and analyzed by sequencing. The sequence revealed that the shorter PCR fragment had a deletion of 153 base pairs, corresponding to nucleotides 904 to 1055 of SEQ ID NO: 7. These deleted bases correspond to exon 4 of the human and mouse VEGF-C genes, schematically depicted in FIGS. 13A and 13B. Deletion of exon 4 results in a frameshift, which in turn results in a C-terminal truncation of the full-length VEGF-C precursor, with fifteen amino acid residues translated from exon 5 in a different frame than the frame used to express the full-length protein. Thus, the C-terminal amino acid sequence of the resulting truncated polypeptide would be —Leu (181)-Ser-Lys-Thr-Val-Ser-Gly-Ser-Glu-Glu-Asp-Leu-Pro-His-Glu-Leu-His-Val-Glu (199) (SEQ ID NO: 51). The polypeptide encoded by this splice variant would not contain the C-terminal cleavage site of the VEGF-C precursor. Thus, a putative alternatively spliced RNA form lacking conserved exon 4 was identified in HT-1080 fibrosarcoma cells and this form is predicted to encode a protein of 199 amino acid residues, which could be an antagonist of VEGF-C.

#### EXAMPLE 33

##### VEGF-C is Similarly Processed in Different Cell Cultures in Vitro

To study whether VEGF-C is similarly processed in different cell types, 293 EBNA cells, COS-1 cells and

HT-1080 cells were transfected with wild type human VEGF-C cDNA and labelled with Pro-Mix™ as described in Example 22. The conditioned media from the cultures were collected and subjected to immunoprecipitation using antiserum 882 (described in Example 21, recognizing a peptide corresponding to amino acids 104–120 of SEQ ID NO: 8). The immunoprecipitated polypeptides were separated via SDS-PAGE, and detected via autoradiography. The major form of secreted recombinant VEGF-C observed from all cell lines tested is a 29/32 kD doublet. These two polypeptides are bound to each other by disulfide bonds, as described in Example 22. A less prominent band of approximately 21 kD also was detected in the culture media. Additionally, a non-processed VEGF-C precursor of 63 kDa was observed. This form was more prominent in the COS-1 cells, suggesting that proteolytic processing of VEGF-C in COS cells is less efficient than in 293 EBNA cells. Endogenous VEGF-C (in non-transfected cells) was not detectable under these experimental conditions in the HT-1080 cells, but was readily detected in the conditioned medium of the PC-3 cells. Analysis of the subunit polypeptide sizes and ratios in PC-3 cells and 293 EBNA cells revealed strikingly similar results: the most prominent form was a doublet of 29/32 kDa and a less prominent form the 21 kD polypeptide. The 21 kD form produced by 293 EBNA cells was not recognized by the 882 antibody in the Western blot, although it is recognized when the same antibody is used for immunoprecipitation (see data in previous examples). As reported in Example 21, cleavage of the 32 kD form in 293 EBNA cells occurs between amino acid residues 111 and 112 (SEQ ID NO: 8), downstream of the cleavage site in PC-3 cells (between residues 102 and 103). Therefore, the 21 kD form produced in 293 EBNA cells does not contain the complete N-terminal peptide used to generate antiserum 882. In a related experiment, PC-3 cells were cultured in serum-free medium for varying periods of time (1–8 days) prior to isolation of the conditioned medium. The conditioned medium was concentrated using a Centricon device (Amicon, Beverly, USA) and subjected to Western blotting analysis using antiserum 882. After one day of culturing, a prominent 32 kD band was detected. Increasing amounts of a 21–23 kD form were detected in the conditioned media from 4 day and 8 day cultures. The diffuse nature of this polypeptide band, which is simply called the 23 kD polypeptide in example 5 and several subsequent examples, is most likely due to a heterogeneous and variable amount of glycosylation. These results indicate that, initially, the cells secrete a 32 kD polypeptide, which is further processed or cleaved in the medium to yield the 21–23 kD form. The microheterogeneity of this polypeptide band would then arise from the variable glycosylation degree and, from microheterogeneity of the processing cleavage sites, such as obtained for the amino terminus in PC-3 and 293 EBNA cell cultures. The carboxyl terminal cleavage site could also vary, examples of possible cleavage sites would be between residues 225–226, 226–227 and 227–228 as well as between residues 216–217. Taken together, these data suggest the possibility that secreted cellular protease(s) are responsible for the generation of the 21–23 kD form of VEGF-C from the 32 kD polypeptide. Such proteases could be used in vitro to cleave VEGF-C precursor proteins in solution during the production of VEGF-C, or used in cell culture and in vivo to release biologically active VEGF-C.

#### EXAMPLE 34

##### Differential Binding of VEGF-C Forms by the Extracellular Domains of VEGFR-3 and VEGFR-2

In two parallel experiments, 293 EBNA cells were transfected with a construct encoding recombinant wild type

VEGF-C or a construct encoding VEGF-C  $\Delta$ NACHis (Example 28) and about 48 hours after transfection, metabolically labelled with Pro-Mix™ as described in previous examples. The media were collected from mock-transfected and transfected cells and used for receptor binding analyses.

Receptor binding was carried out in binding buffer (PBS, 0.5% BSA, 0.02% Tween 20, 1 microgram/ml heparin) containing approximately 0.2 microgram of either (a) a fusion protein comprising a VEGFR-3 extracellular domain fused to an immunoglobulin sequence (VEGFR-3-Ig) or (b) a fusion protein comprising VEGFR-2 extracellular domain fused to an alkaline phosphatase sequence (VEGFR-2-AP; Cao et al., *J. Biol. Chem.* 271:3154–62 (1996)). As a control, similar aliquots of the 293 EBNA conditioned media were mixed with 2  $\mu$ l of anti-VEGF-C antiserum (VEGF-C IP).

After incubation for 2 hours at room temperature, anti-VEGF-C antibodies and VEGFR-3-Ig protein were adsorbed to protein A-sepharose (PAS) and VEGFR-2-AP was immunoprecipitated using anti-AP monoclonal antibodies (Medix Biotech, Genzyme Diagnostics, San Carlos, Calif., USA) and protein G-sepharose. Complexes containing VEGF-C bound to VEGFR-3-Ig or VEGFR-2-AP were washed three times in binding buffer, twice in 20 mM Tris-HCl (pH 7.4) and VEGF-C immunoprecipitates were washed three times in RIPA buffer and twice in 20 mM Tris-HCl (pH 7.4) and analyzed via SDS-PAGE under reducing and nonreducing conditions. As a control, the same media were precipitated with anti-AP and protein G-sepharose (PGS) or with PAS to control for possible nonspecific adsorption.

These experiments revealed that VEGFR-3 bound to both the 32/29 kD and 21–23 kD forms of recombinant VEGF-C, whereas VEGFR-2 bound preferentially to the 21–23 kD component from the conditioned media. In addition, small amounts of 63 kD and 52 kD VEGF-C forms were observed binding with VEGFR-3. Further analysis under nonreducing conditions indicates that a great proportion of the 21–23 kD VEGF-C bound to either receptor does not contain inter-chain disulfide bonds. These findings reinforce the results that VEGF-C binds VEGFR-2. This data suggests a utility for recombinant forms of VEGF-C which are active towards VEGFR-3 only or which are active towards both VEGFR-3 and VEGFR-2. On the other hand, these results, together with the results in Example 28, do not eliminate the possibility that the 32/29 kD dimer binds VEGFR-3 but does not activate it. The failure of the 32/29 kD dimer to activate VEGFR-3 could explain the finding that conditioned medium from the N-His VEGF-C transfected cells induced a less prominent tyrosine phosphorylation of VEGFR-3 than medium from VEGF-C  $\Delta$ NACHis transfected cells, even though expression of the former polypeptide was much higher. Stable VEGF-C polypeptide mutants that bind to a VEGF-C receptor but fail to activate the receptor are useful as VEGF-C antagonists.

#### EXAMPLE 35

##### Discovery of VEGF-C Analogs That Selectively Bind to and Activate VEGFR-3, But Not VEGFR-2

To further identify the cysteine residues of VEGF-C that are critical for retaining VEGF-C biological activities, an additional VEGF-C mutant, designated VEGF-CANACHisC156S, was synthesized, in which the cysteine residue at position 156 of the 419 amino acid VEGF-C precursor (SEQ ID NO: 8; Genbank accession number X94216) was replaced with a serine residue.

The mutagenesis procedure was carried out using the construct of VEGF-CANACHis (see Example 28), cloned in the pALTER vector, and the Altered sites II in vitro mutagenesis system of Promega. An oligonucleotide 5'-GACGGACACAGATGGAGGTTAAAG-3' (SEQ ID NO: 52) was used to introduce the desired mutation in the CDNA encoding VEGF-CANACHis. The resulting mutated VEGF-C cDNA fragment was subcloned into the HindIII/NcoI sites of the pREP-7 vector (Invitrogen), and the final construct was resequenced to confirm the C156S mutation. The resultant clone has an open reading frame encoding amino acids 103-225 of SEQ ID NO: 8 (with a serine codon at position 156), and further encoding a 6xHis tag.

The wildtype VEGF-C cDNA and three VEGF-C mutant constructs (VEGF-C R226,227S, VEGF-C  $\Delta$ NACHis, and VEGF-C  $\Delta$ NACHisC156S) were used to transfect 293 EBNA cells, which were subcultured 16 hours after transfection. About 48 hours after transfection, the media were changed to DMEM/0.1% BSA, and incubation in this medium was continued for an additional 48 hours. The resultant conditioned media were concentrated 30-fold using Centrprep-10 (Amicon), and the amount of VEGF-C in the media was analyzed by Western blotting using the anti-VEGF-C antiserum 882 for immunodetection. Different amounts of the recombinant VEGF-C  $\Delta$ NACHis, purified from a yeast expression system, were analyzed in parallel as reference samples to measure and equalize the VEGF-C concentrations in the conditioned media. The conditioned medium from mock-transfected cells was used to dilute the VEGF-C conditioned media to achieve equal concentrations.

An aliquot of the transfected cells were metabolically labelled for 6 h with 100 microcuries/ml of the PRO-MIX<sup>TM</sup> L- [<sup>35</sup>S] in vitro cell labelling mix (Amersham). The conditioned media were collected, and binding of the radioactively labelled VEGF-C proteins to the extracellular domains of VEGFR-3 and VEGFR-2 was analyzed using recombinantly produced VEGFR-3EC-Ig and VEGFR-2EC-Ig constructs (containing seven and three Ig loops of the extracellular domains of the respective receptors, fused to an immunoglobulin heavy chain constant region).

As shown in FIG. 14A, all processed VEGF-C forms secreted to the culture medium bound to VEGFR-3EC domain, with preferential binding of the 21 kDa form (left panel). When present at high concentrations, the VEGF-C forms of 58 kDa and 29/31 kDa bound to some extent non-specifically to protein A Sepharose (PAS, right panel).

The VEGFR-2EC domain preferentially bound the mature 21 kDa form of wildtype VEGF-C and VEGF-CANACHis. Significantly, VEGF-CANACHisC156S failed to bind the VEGFR2-EC (FIG. 14A, middle panel).

Next, the ability of the above-described VEGF-C polypeptides to compete with the <sup>125</sup>I-VEGF-CANACHis for binding to VEGFR-2 and VEGFR-3 was analyzed. Scatchard analysis using VEGF-C  $\Delta$ CANHis provided indications of the VEGF-C binding affinity for VEGFR-3 ( $K_D$ =135 pM) and VEGFR-2 ( $K_D$ =410 pM). Ten micrograms of the purified yeast VEGF-C  $\Delta$ NACHis was labeled using 3 mCi of Iodine-125, carrier-free (Amersham), and an Iodogen Iodination Reagent (Pierce), according to the standard protocol of Pierce. The resulting specific activity of the labeled VEGF-CANACHis was  $1.25 \times 10^5$  cpm/ $\mu$ g.

To study receptor binding, PAE/VEGFR-2 and PAE/VEGFR-3 cells were seeded into 24-well tissue culture plates (Nunc), which had been coated with 2% gelatin in PBS. The <sup>125</sup>I-VEGF-C  $\Delta$ NACHis ( $2 \times 10^5$  cpm) and different

amounts of media containing equal concentrations of the non-labeled VEGF-C (wildtype and mutants) were added to each plate in Ham's F12 medium, containing 25 mM HEPES (pH 8.0), 0.1% BSA, and 0.1% NaN<sub>3</sub>. The binding was allowed to proceed at room temperature for 90 minutes. The plates were then transferred onto ice and washed three times with ice-cold PBS containing 0.1% BSA. The cells were then lysed in 1 M NaOH, the lysates were collected, and the radioactivity was measured using a  $\gamma$ -counter. Binding in the presence of VEGF-C-containing conditioned medium was calculated as a percentage of binding observed in parallel control studies wherein equal volumes of medium from mock-transfected cells were used instead of VEGF-C conditioned media.

As shown in FIG. 14B, left panel, all VEGF-C mutants displaced <sup>125</sup>I-VEGF-CANACHis from VEGFR-3. The efficiency of displacement was as follows: VEGF-CANACHisC156S > VEGF-CANACHis > wildtype VEGF-C > VEGF-CR226,227S. These results indicate that enhanced binding to VEGFR-3 was obtained upon "recombinant maturation" of VEGF-C. Recombinant VEGF165 failed to displace VEGF-C from VEGFR-3.

VEGF, VEGF-CANACHis, and wildtype VEGF-C all efficiently displaced labeled VEGF-CANACHis from VEGFR-2, with VEGF-CANACHis being more potent when compared to wildtype VEGF-C (FIG. 1B, right panel). The non-processed VEGF-C R226,227S showed only weak competition of <sup>125</sup>I-VEGF-CANACHis.

Surprisingly, VEGF-CANACHisR156S failed to displace VEGF-CANACHis from VEGFR-2, thus confirming the above described results obtained using a soluble extracellular domain of VEGFR-2.

The ability of the above mentioned VEGF-C forms to stimulate tyrosine phosphorylation of VEGFR-3 and VEGFR-2 was also investigated. Importantly, identical dilutions of the conditioned media were used for these experiments and for the competitive binding experiments described above. A Western blot analysis of the conditioned media using anti-VEGF-C antiserum 882 was performed to confirm the approximately equal relative amounts of the factors present.

The stimulation of VEGFR-3 and VEGFR-2 autophosphorylation by the different VEGF-C forms in general correlated with their binding properties, as well as with the degree of "recombinant processing" of VEGF-C. The VEGF-CANACHisC156S appeared to be at least as potent as VEGF-CANACHis in stimulating VEGFR-3 autophosphorylation. VEGF-CANACHis showed a higher potency when compared to wildtype VEGF-C in its ability to stimulate tyrosine autophosphorylation of both VEGFR-2 and VEGFR-3. The VEGF-CR226,227S conditioned medium possessed a considerably weaker effect on autophosphorylation of VEGFR-3, and almost no effect on VEGFR-2 autophosphorylation.

Stimulation of VEGFR-2 tyrosine phosphorylation by VEGF-CANACHisC156S did not differ from that of conditioned medium from the mock transfected cells, thus confirming the lack of VEGFR-2-binding and VEGFR-2-activating properties of this mutant.

The ability of VEGF-C  $\Delta$ NACHisC156S to alter vascular permeability in vivo was analyzed using the Miles assay (see Example 29). The recombinant VEGF-C forms assayed ( $\Delta$ NACHis,  $\Delta$ NACHisC156S) were produced by 293 cells, purified from conditioned media using Ni-NTA Superflow resin (QIAGEN) as previously described, and pretreated with 15  $\mu$ g/ml of anti-human VEGF neutralizing antibody

(R&D systems) to neutralize residual amounts of co-purified, endogenously produced VEGF. Eight picomoles of the various VEGF-C forms, as well as 2 pmol of recombinant human VEGF165 (R&D systems) and approximately 2 pmol of VEGF165 from the conditioned medium which were either non-treated or pretreated with the above mentioned VEGF-neutralizing antibody were injected subcutaneously to the back region of a guinea pig. The area of injection was analyzed 20 minutes after injections. Both VEGF and VEGF-C  $\Delta$ NACHis caused increases in vascular permeability, whereas  $\Delta$ NACHisC156S did not affect vascular permeability. The neutralizing antibody completely blocked permeability activity of VEGF but did not affect VEGF-C activity. Residual permeability activity observed for the VEGF-containing conditioned medium even after its treatment with VEGF neutralizing antibody was presumably caused by permeability factors other than VEGF that are produced by 293 cells.

The Miles assay also was used to assay the ability of VEGF-C R226,227S (8 pM, pretreated with anti-VEGF antibody) to induce vascular permeability. The results indicated that the ability of VEGF-C R226,227S to induce vascular permeability was much weaker when compared to wildtype and  $\Delta$ NACHis forms of VEGF-C. Collectively, this Miles assay data is consistent with the VEGFR-2 binding and autophosphorylation data described above, and indicates that VEGF-C effect on vascular permeability is mediated via VEGFR-2.

The foregoing data indicates that proteolytic processing of VEGF-C results in an increase in its ability to bind and to activate VEGFR-3 and VEGFR-2. Non-processed VEGF-C is a ligand and an activator of preferentially VEGFR-3, while the mature 21/23 kDa VEGF-C form is a high affinity ligand and an activator of both VEGFR-3 and VEGFR-2.

Moreover, replacement of the cysteine residue at position 156 (of prepro-VEGF-C, SEQ ID NO: 8) creates a selective ligand and activator of VEGFR-3. This alteration inactivates the ability of processed VEGF-C to bind to VEGFR-2 and to activate VEGFR-2. Importantly, it is believed that the elimination of the cysteine at position 156 is the alteration responsible for this unexpected alteration in VEGF-C selectivity, and not the substitution of a serine per se. It is expected that replacement of the cysteine at position 156 with other amino acids, or the mere deletion of this cysteine, will also result in VEGF-C analogs having selective biological activity with respect to VEGFR-3. All such replacement and deletion analogs (collectively referred to as VEGF-C  $\Delta$ C<sub>156</sub> polypeptides) are contemplated as aspects of the present invention.

VEGF-C polypeptides that have the C156S mutation (or functionally equivalent mutations at position 156) and that retain biological activity with respect to VEGFR-3, such as VEGF-C  $\Delta$ NACHisC156S, are useful in all of the same manners described above for wildtype VEGF-C proteins and biologically active fragments thereof where VEGFR-3 stimulation is desired. It is contemplated that most biologically active VEGF-C fragments and processing variants, including but not limited to the biologically active fragments and variants identified in preceding examples, will retain VEGF-C biological activity (as mediated through VEGFR-3) when a  $\Delta$ C<sub>156</sub> mutation is introduced. All such biologically active VEGF-C  $\Delta$ C<sub>156</sub> polypeptides are intended as an aspect of the present invention.

Moreover, VEGF-C forms containing the C156S mutation or equivalent mutations can be used to distinguish those effects of VEGF-C mediated via VEGFR-3 and VEGFR-2

from those obtained via only VEGFR-3. The ability of such VEGF-C polypeptides to selectively stimulate VEGFR-3 are also expected to be useful in clinical practice, it being understood that selectivity of a pharmaceutical is highly desirable in many clinical contexts. For example, the selectivity of VEGF-C  $\Delta$ C<sub>156</sub> polypeptides for VEGFR-3 binding suggests a utility for these peptides to modulate VEGF-C biological activities mediated through VEGFR-3, without significant concomitant modulation of blood vessel permeability or other VEGF-C activities that are modulated through VEGFR-2.

The data presented herein also indicates a utility for  $\Delta$ C<sub>156</sub> polypeptides that are capable of binding VEGFR-3, but that do not retain biological activity mediated through VEGFR-3. Specifically, such forms are believed to be capable of competing with wildtype VEGF-C for binding to VEGFR-3, and are therefore contemplated as molecules that inhibit VEGF-C-mediated stimulation of VEGFR-3. Because of the  $\Delta$ C<sub>156</sub> alteration, such polypeptides (especially covalent or noncovalent dimers of such polypeptides) are not expected to bind VEGFR-2. Thus, certain  $\Delta$ C<sub>156</sub> polypeptides and polypeptide dimers are expected to have utility as selective inhibitors of VEGF-C biological activity mediated through VEGFR-3 (i.e., without substantially altering VEGF-C mediated stimulation of VEGFR-2).

In another embodiment of the invention, heterodimers comprising a biologically active VEGF-C polypeptide in association with a  $\Delta$ C<sub>156</sub> polypeptide are contemplated. It is contemplated that such heterodimers can be formed in vitro, as described below in Example 37, or formed in vivo with endogenous VEGF-C following administration of a  $\Delta$ C<sub>156</sub> polypeptide. Such heterodimers are contemplated as modulators of VEGF-C mediated effects in cells where the biological effects of VEGF-C are mediated through VEGFR-2/VEGFR-3 heterodimers. VEGF-C  $\Delta$ C<sub>156</sub> polypeptides in homodimers or in heterodimers with wt VEGF-C might selectively inhibit the ability of the latter to induce VEGF-like effects, particularly to increase the vascular permeability.

### EXAMPLE 36

#### Utility for VEGF-C in Promoting Myelopoiesis

The effects of VEGF-C on hematopoiesis were also analyzed. Specifically, leukocytes populations were analyzed in blood samples taken from the F1 transgenic mice described in Example 29, and from their non-transgenic littermates. Leukocyte population data from these mice and from non-transgenic FVB-NIH control mice (i.e., the strain used to generate the transgenic mice) are set forth in the tables below.

Cell Type	FVB/NIH MICE				mean $\pm$ SD
	male 5.5 months	male 5.5 months	female 9.5 months	male 9.5 months	
Lymphocytes	72.20%	82.17%	84.25%	74.25%	78.22 $\pm$ 5.10
Neutrophils	23.00%	15.17%	14.25%	22.25%	18.67 $\pm$ 3.98
Monocytes	0.65%	1.00%	0.25%	0.50%	0.60 $\pm$ 0.27
Eosinophils	2.15%	1.70%	1.25%	3.00%	2.03 $\pm$ 0.65
Basophils	0.00%	0.00%	0.00%	0.00%	0 $\pm$ 0

-continued

VEGF-C TRANSGENIC MICE				
Cell Type	male 2 months	male 3.5 months	male 7 months	mean $\pm$ $\sigma$
Lymphocytes	41.3%	41.50%	18.70%	33.83 $\pm$ 10.70
Neutrophils	55.3%	53.80%	80.17%	63.09 $\pm$ 12.09
Monocytes	2.16%	1.30%	0.67%	1.38 $\pm$ 0.6
Eosinophils	1.17%	3.50%	.50%	1.72 $\pm$ 1.29
Basophils	0.00%	0.00%	0.00%	0 $\pm$ 0

VEGF-C NEGATIVE CONTROL MICE (NON-TRANSGENIC LITTERMATES OF VEGF-C TRANSGENIC MICE)					
Cell Type	male 2 months	male 2 months	male 3.5 months	male 7 months	mean $\pm$ $\sigma$
Lymphocytes	89.00%	67.50%	91.00%	71.30%	79.7 $\pm$ 10.4
Neutrophils	7.75%	23.00%	7.00%	23.75%	15.38 $\pm$ 8.01
Monocytes	1.50%	0.50%	0.83%	0.75%	0.90 $\pm$ 0.37
Eosinophils	1.50%	9.00%	0.67%	4.00%	3.79 $\pm$ 3.25
Basophils	0.00%	0.00%	0.50%	0.50%	0.25 $\pm$ 0.25

As the foregoing data indicates, the overexpression of VEGF-C in the skin of the transgenic mice correlates with a distinct alteration in leukocyte populations. Notably, the measured populations of neutrophils were markedly increased in the transgenic mice. One explanation for the marked increase in neutrophils is a myelopoietic activity attributable to VEGF-C. A VEGF-C influence on leukocyte trafficking in and out of tissues also may effect observed neutrophil populations. Fluorescence-activated cell sorting analysis, performed on isolated human bone marrow and umbilical cord blood CD34-positive hematopoietic cells, demonstrated that a fraction of these cells are positive for Flt4 (VEGFR-3). Thus, the VEGF-C effect on myelopoiesis may be exerted through this VEGFR-3-positive cell population and its receptors. In any case, the foregoing data indicates a use for VEGF-C polypeptides to increase granulocyte (and, in particular, neutrophil) counts in human or non-human subjects, i.e., in order to assist the subject fight infectious diseases. The exploitation of the myelopoietic activity of VEGF-C polypeptides is contemplated both in vitro (i.e., in cell culture) and in vivo, as a sole myelopoietic agent and in combination with other effective agents (e.g., granulocyte colony stimulating factor (G-CSF)).

Additional studies of the myelopoietic effect of VEGF-C, using VEGF-C mutants (e.g., VEGF-C  $\Delta C_{156}$  polypeptides, VEGF-C  $\Delta N\Delta C$ His, VEGF-C R226,227S) having altered VEGFR-2 binding affinities, will elucidate whether this effect is mediated through VEGFR-2, VEGFR-3, or both receptors, for example. The results of such analysis will be useful in determining which VEGF-C mutants have utility as myelopoietic agents and which have utility as agents for inhibiting myelopoiesis.

#### EXAMPLE 37

##### Generation of Heterodimers Consisting of Members of the VEGF Family of Growth Factors

Both naturally-occurring and recombinantly-produced heterodimers of polypeptides of the PDGF/VEGF family of growth factors have been shown to exist in nature and possess mitogenic activities. See, e.g., Cao et al., *J. Biol. Chem.*, 271:3154-62 (1996); and DiSalvo, et al., *J. Biol. Chem.*, 270:7717-7723 (1995). Heterodimers comprising a VEGF-C polypeptide may be generated essentially as

described in Cao et al. (1996), using recombinantly produced VEGF-C polypeptides, such as the VEGF-C polypeptides described in the preceding examples. Briefly, a recombinantly produced VEGF-C polypeptide is mixed at an equimolar ratio with another recombinantly produced polypeptide of interest, such as a VEGF, VEGF-B, PLGF, PDGF $\alpha$ , PDGF $\beta$ , or c-fos induced growth factor polypeptide. (See, e.g., Cao et al. (1990); Collins et al., *Nature*, 316:748-750 (1985) (PDGF- $\beta$ , GenBank Acc. No. X02811); Claesson-Welsh et al., *Proc. Natl. Acad. Sci. USA*, 86(13):4917-4921 (1989) (PDGF- $\alpha$ , GenBank Acc. No. M22734); Claesson-Welsh et al., *Mol. Cell. Biol.*, 8:3476-3486 (1988) (PDGF- $\beta$ , GenBank Acc. No. M21616); Olofsson et al., *Proc. Natl. Acad. Sci. (USA)*, 93:2576-2581 (1996) (VEGF-B, GenBank Acc. No. U48801); Maglione et al., *Proc. Natl. Acad. Sci. (USA)*, 88(20):9267-9271 (1996) (PLGF, GenBank Acc. No. X54936); Heldin et al., *Growth Factors*, 8:245-252 (1993); Folkman, *Nature Med.*, 1:27-31 (1995); Friesel et al., *FASEB J.*, 9:919-25 (1995); Mustonen et al., *J. Cell. Biol.*, 129:895-98 (1995); Orlandini, S., *Proc. Natl. Acad. Sci. USA*, 93(21):11675-11680 (1996); and others cited elsewhere herein. The mixed polypeptides are incubated in the presence of guanidine-HCl and DTT. The thiol groups are then protected with S-sulfonation, and the protein is dialyzed overnight, initially against urea/glutathione-SH, glutathione-S-S-glutathione, and subsequently against 20 mM Tris-HCl.

In a preferred embodiment, a variety of differently processed VEGF-C forms and VEGF-C variants and analogs, such as the ones described in the preceding examples, are employed as the VEGF-C polypeptide used to generate such heterodimers. Thereafter, the heterodimers are screened to determine their binding affinity with respect to receptors of the VEGF/PDGF family (especially VEGFR-1, VEGFR-2, and VEGFR-3), and their ability to stimulate the receptors (e.g., assaying for dimer-stimulated receptor phosphorylation in cells expressing the receptor of interest on their surface). The binding assays may be competitive binding assays such as those described herein and in the art. In the initial binding assays, recombinantly produced proteins comprising the extracellular domains of receptors are employable, as described in preceding examples for VEGFR-2 and VEGFR-3. Heterodimers that bind and stimulate receptors are useful as recombinant growth factor polypeptides. Heterodimers that bind but do not stimulate receptors are useful as growth factor antagonists. Heterodimers that display agonistic or antagonistic activities in the screening assays are further screened using, e.g., endothelial cell migration assays, vascular permeability assays, and in vivo assays. It will also be apparent from the preceding examples that dimers comprising two VEGF-C polypeptides (i.e., dimers of identical VEGF-C polypeptides as well as dimers of different VEGF-C polypeptides) are advantageously screened for agonistic and antagonistic activities using the same assays.

In one preferred embodiment, VEGF-C  $\Delta C_{156}$  polypeptide is employed to make the dimers. It is anticipated that agonists and antagonists comprising a VEGF-C  $\Delta C_{156}$  polypeptide will have increased specificity for stimulating and inhibiting VEGFR-3, without concomitant stimulation or inhibition of VEGFR-2.

In another preferred embodiment, VEGF-C polypeptides wherein the C-terminal proteolytic cleavage site has been altered to reduce or eliminate C-terminal processing (e.g. VEGF-C R226,227S) is employed to make dimers for screening for inhibitory activity.

In yet another preferred embodiment, VEGF-C polypeptides comprising amino-terminal fragments (e.g., the VEGF-C 15 kD form described herein) of VEGF-C are employed to make dimers.

It is further contemplated that inactivation of only one polypeptide chain in a dimer could be enough to generate an inhibitory molecule, which is demonstrated e.g., by the generation of PDGF inhibitory mutant as reported in Vassbotn, *Mol. Cell. Biol.*, 13:4066-4076 (1993). Therefore, in one embodiment, inhibition is achieved by expression in vivo of a polynucleotide (e.g., a cDNA construct) encoding the heterodimerization partner which is unable to bind (or binds inefficiently) to the receptor, or by direct administration of that monomer in a pharmaceutical composition.

#### EXAMPLE 38

##### Formation and Screening of Useful Recombinant VEGF/VEGF-C Genes and Polypeptides

Amino acid sequence comparison reveals that mature VEGF-C bears structural similarity to VEGF121 [Tischer et al., *J. Biol. Chem.*, 266(18):11947-54 (1991)], with certain noteworthy structural differences. For example, mature VEGF-C contains an unpaired cysteine (position 137 of SEQ ID NO: 8) and is able to form non-covalently bonded polypeptide dimers. In one embodiment of the invention, a VEGF analog is created wherein the unpaired cysteine residue from mature VEGF-C is introduced at an analogous position of VEGF (e.g., introduced at Leu<sub>58</sub> of the human VEGF165 precursor (FIG. 2, Genbank Acc. No. M32977) to generate a VEGF<sup>rev</sup> mutant designated VEGF L58C). Such an alteration is introduced into the VEGF165 coding sequence using site-directed mutagenesis procedures known in the art, such as the procedures described above in preceding examples to generate various VEGF-C mutant forms. This VEGF<sup>rev</sup> mutant is recombinantly expressed and is screened (alone and as a heterodimer with other VEGF and VEGF-C forms) for VEGFR-2 and/or VEGFR-3 binding, stimulatory, and inhibitory activities, using in vitro and in vivo activity assays as described elsewhere herein. To form

another VEGF analog of the invention, a VEGF<sup>rev</sup> mutant is altered to remove a conserved cysteine corresponding to cyst of the VEGF165 precursor. Elimination of this cysteine from the VEGF L58C would result in a VEGF analog resembling VEGF- $\Delta$ NACHisC156S. This VEGF analog is screened for its VEGF-inhibitory activities with respect to VEGFR-2 and/or VEGFR-1 and for VEGF-C like stimulatory or inhibitory activities.

Another noteworthy structural difference between VEGF and VEGF-C is the absence in VEGF-C of several basic residues found in VEGF (e.g., residues Arg<sub>108</sub>, Lys<sub>110</sub> and His<sub>112</sub> in the VEGF165 precursor shown in FIG. 2) that have been implicated in VEGF receptor binding. See Keyt et al., *J. Biol. Chem.*, 271(10):5638-46 (1996). In another embodiment of the invention, codons for basic residues (lys, arg, his) are substituted into the VEGF-C coding sequence at one or more analogous positions by site-directed mutagenesis. For example, in a preferred embodiment, Glu<sub>87</sub>, Thr<sub>189</sub>, and Pro<sub>191</sub>, in VEGF-C (SEQ ID NO: 8) are replaced with Arg, Lys, and His residues, respectively. The resultant VEGF-C analogs (collectively termed "VEGF-C<sub>basic</sub>" polypeptides) are recombinantly expressed and screened for VEGFR-1, VEGFR-2, and VEGFR-3 stimulatory and inhibitory activity. The foregoing VEGF and VEGF-C analogs that have VEGF-like activity, VEGF-C-like activity, or that act as inhibitors of VEGF or VEGF-C, are contemplated as additional aspects of the invention. Polynucleotides encoding the analogs also are intended as aspects of the invention.

Deposit of Biological Materials: Plasmid FL14-L has been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Dr., Rockville Md. 20952 (USA), pursuant to the provisions of the Budapest Treaty, and has been assigned a deposit date of Jul. 24, 1995 and ATCC accession number 97231.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those in the art. Accordingly, only such limitations as appear in the appended claims should be placed on the invention.

#### SEQUENCE LISTING

##### (1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 57

##### (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 4416 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CACGGAGSAG TCACACGTC TCGACACCGG TGACAGCCTG TCATCTCTCT GCAGGGGACA    180
GCACCCCTC GAGTGGGCTT GSCCAGSAGC TCAGGAGGCG CAGCCACCG GAGACAAGGA    240
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GTGGGTGCCC TGTGTGGTGT CCATCCCCGG CCTCAATGTC ACGCTGCCCT CGCAAAGCTC	540
GSTGCTGTGG CCAGACGGGC AGGAGGTGGT GTGGGATGAC CGGCGGGGCA TGCTCGTGTG	600
CACGCCACTG CTGCACGATG CCTGTACCT GCAGTGCSAG ACCACCTGGG GAGACCAGGA	660
CTTCTTTCC AACCCCTTC TGTGACAT CACAGGCAAC GAGCTCTATG ACATCCAGCT	720
GTGGCCAGG AAGTCGCTGG AGCTGTGGT AGGGGAGAAG CTGGTCCTGA ACTGCCCGT	780
GTGGGCTGAG TTAACTCAG GTGTACCTT TGA CTGGGAC TACCCAGGA AGCAGGCAGA	840
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CCTGACATC CACAAGTCA GCGAGCAGGA CCTGGGCTCG TATGTGTGCA AGGCCAACAA	960
CGGCATCCAG CGATTTCGG AGAGCACCGA GGTCATCTG CATGAAAATC CCTTCATCAG	1020
CCTGAGTGG CTCGAAGGAC CCATCTGGA GGCACGGCA GGAGACGAGC TGGTGAAGCT	1080
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GCAGGATGCC GTGAACCCCA TCGAGAGCT GGACACCTGG ACCGAGTTG TGGAGGAAA	1560
GAATAAGACT GTGAGCAAG TGTGATCCA GAATGCCAAC GTGCTTGCCA TGTACAAGTG	1620
TGTGGTCTC AACAAAGTGG GCCAGGATGA GCGGCTCATC TACTTCTATG TGACCACCAT	1680
CCCCGACGGC TTCACCATG AATCAAGCC ATCCGAGSAG CTACTAGAGG GCCAGCCGGT	1740
GCTCTGAGC TGCCAAGCG ACAGCTACAA GTACGAGCAT CTGCCCTGGT ACCGCCCTCA	1800
CCTGTCCAG CTGCACGATG CGCACGGGA CCGCTTCTG CTGACTGCA AGAAGCTGCA	1860
TCTGTGCCC ACCCTCTGG CCGCCAGCT GGAGGAGGTG GCACCTGGGG CGCGCCACGC	1920
CACGCTCAGC CTGAGTATC CCGCGTCCG GCGCGAGCAC GAGGGCCACT ATGTGTGCGA	1980
AGTGCAAGAC CGGCGCAGCC ATGACAAGCA CTGCCACAAG AAGTACCTGT CGGTGCAGGC	2040
CCTGGAAGCC CCTGGCTCA CGCAGAATG GACCGACCTC CTGGTGAACG TGAGCGACTC	2100
GCTGAGATG CAGTGTGG TGGCCGAGC GCACGCGGCC AGCATCGTGT GGTACAAAGA	2160
CGAGAGGCTG CTGAGGAAA AGCTGGAGT CGACTTGGCG GACTCCAACC AGAAGCTGAG	2220
CATCCAGCGC GTGCCGAGG AGGATGCGG ACCTATCTG TGCAAGTGT GCAACGCCAA	2280
GGGCTGCGTC AACTCTCCG CCAGCCTGGC CGTGAAGGC TCCGAGGATA AGGGCAGCAT	2340
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## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 216 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CAAGAAAGCG GCTTCAGCTG TAAAGGACCT GCCAGAATG TGGCTGTGAC CAGGGCACAC	60
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## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 4273 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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 ACCTGCAATTA ATGAATCGGC CAACGCGCGG GGAGAGCGCG TTTGCGTATT GGGCGCTCTT 1200  
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CTGTGCTTA TTGAACTAA CCAATCAGT CGCTTCTGCG TTCTGTGCG GCGCTTCTGC	4200
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4273

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Pro Met Thr Pro Thr Thr Tyr Lys Gly Ser Val Asp Asn Gln Thr Asp  
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 Ser Gly Met Val Leu Ala Ser Glu Glu Phe Glu Gln Ile Glu Ser Arg  
 20 25 30  
 His Arg Gln Glu Ser Gly Phe Arg  
 35 40

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Xaa Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr Asn Thr Glu Ile  
 : 5 10 15  
 Leu Lys

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 219 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCACTATAGG GAGACCCAAG CTTGGTACCG AGCTCGGATC CACTAGTAAC GGCCGCCAGT 60  
 GTGGTGGAAI TCGACGAACT CATGACTGTA CTCTACCCAG AATATTGGAA AATGTACAAG 120  
 TGTCAGCTAA GCGAAGGAGG CTGGCAACAT AACAGAGAAC AGGCCAACCT CAACTCAAGG 180  
 ACAGAAGAGA CTATAAAATT CGCTGCAGCA CACTACAAC 219

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1997 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 352..1608

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:	
CCCCCCCCCG CTCTCCAAAA AGGTACACCG ACGCGGACCG CGGCGGCGCT CTCGCTCGCC	60
CTCGCTTCAC CTCGCGGGGT CCGAATGCGG GGAGCTCGGA TGTGCGGTTT CCTGTGAGGC	120
TTTACGTGA CACCGCCCGC CTTCGCCCGG CACTGGCTGG GAGGCGCGCC TCGAAATTTG	180
CGAACGCGGA CCCCCGACCG CGGTCCCGCC GCCTCCGCT CCGCCAGGGG GGGTCGCGCG	240
GAGGAGCCCG GGGGAGAGGG ACCAGGAGCG GCCCGCGGCC TCGCAGGGGC GCCCGCCCGC	300
CCACCCCTGC CCCCAGCAGG GGACCGGTCC CCGACCCCGG GTCCCTCCAC C ATG CAC	357
	Met His
TTG CTG GGC TTC TTC TCT GTG GCG TGT TCT CTG CTC SCC GGT GCG CTG	405
Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala Ala Leu	
5 10 15	
CTC CCG GGT CCT CGC GAG GCG CCC SCC GCC SCC GCC SCC TTC GAG TCC	453
Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Ala Phe Glu Ser	
20 25 30	
GGA CTC GAC CTC TCG GAC GCG GAG CCC GAC GCG GGC GAG GCC ACG GCT	501
Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala Thr Ala	
35 40 45 50	
TAT GCA AGC AAA GAT CTG GAG GAG CAG TTA CGG TCT GTG TCC AGT GTA	549
Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser Ser Val	
55 60 65	
GAT GAA CTC ATG ACT GTA CTC TAC CCA GAA TAT TGG AAA ATG TAC AAG	597
Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met Tyr Lys	
70 75 80	
TGT CAG CTA AGG AAA GGA GGC TGG CAA CAT AAC AGA GAA CAG GCC AAC	645
Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln Ala Asn	
85 90 95	
CTC AAC TCA AGG ACA GAA GAG ACT ATA AAA TTT GCT GCA GCA CAT TAT	693
Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr	
100 105 110	
AAT ACA GAG ATC TTG AAA AGT ATT GAT AAT GAG TGG AGA AAG ACT CAA	741
Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln	
115 120 125 130	
TGC ATG CCA CGS GAG GTG TGT ATA GAT GTG GGG AAG GAG TTT GGA GTC	789
Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Val	
135 140 145	
GCG ACA AAC ACC TTC TTT AAA CCT CCA TGT GTG TCC CTC TAC AGA TGT	837
Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys	
150 155 160	
GCG GGT TGC TGC AAT AGT GAG GCG CTG CAG TGC ATG AAC ACC AGC ACG	885
Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr	
165 170 175	
AGC TAC CTC AGC AAG ACG TTA TTT GAA ATT ACA GTG CCT CTC TCT CAA	933
Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln	
180 185 190	
GSC CCC AAA CCA GTA ACA ATC AGT TTT GCC AAT CAC ACT TCC TGC CGA	981
Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg	
195 200 205 210	
TGC ATG TCT AAA CTG GAT GTT TAC AGA CAA GTT CAT TCC ATT ATT AGA	1029
Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg	
215 220 225	
CGT TCC CTG CCA GCA ACA CTA CCA CAG TGT CAG GCA GCG AAC AAG ACC	1077
Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr	
230 235 240	
TGC CCC ACC AAT TAC ATG TGG AAT AAT CAC ATC TGC AGA TGC CTG GCT	1125
Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala	
245 250 255	

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CAG GAA GAT TTT ATG TTT TCC TCG GAT GCT GGA GAT GAC TCA ACA GAT 1173  
 Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp  
 260 255 270  
 GGA TTC CAT GAC ATC TGT GGA CCA AAC AAG GAG CTG GAT GAA GAG ACC 1221  
 Gly Phe His Asp Ile Cys Gly Phe Asn Lys Glu Leu Asp Glu Glu Thr  
 275 280 285 290  
 TGT CAG TGT GTC TGC AGA GCG GGG CTT CGG CCT GCC AGC TGT GGA CCC 1269  
 Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro  
 295 300 305  
 CAC AAA GAA CTA GAC AGA AAC TCA TGC CAG TGT GTC TGT AAA AAC AAA 1317  
 His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys  
 310 315 320  
 CTC TTC CCC AGC CAA TGT GGG GCC AAC CGA GAA TTT GAT GAA AAC ACA 1365  
 Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr  
 325 330 335  
 TGC CAG TGT GTA TGT AAA AGA ACC TGC CCC AGA AAT CAA CCC CTA AAT 1413  
 Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn  
 340 345 350  
 CCT GGA AAA TGT GCC TGT GAA TGT ACA GAA AGT CCA CAG AAA TGC TTG 1461  
 Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu  
 355 360 365 370  
 TTA AAA GGA AAG AAG TTC CAC CAC CAA ACA TGC AGC TGT TAC AGA CCG 1509  
 Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg  
 375 380 385  
 CCA TGT ACG AAC CGC CAG AAG GCT TGT GAG CCA GGA TTT TCA TAT AGT 1557  
 Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser Tyr Ser  
 390 395 400  
 GAA GAA GTG TGT CGT TGT GTC CCT TCA TAT TGG AAA AGA CCA CAA ATG 1605  
 Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro Gln Met  
 405 410 415  
 AGC TAAGATTGTA CTGTTTCCCA GTTCATCGAT TTTCTATTAT GGAAAACTGT 1658  
 Ser  
 GTTCCACAG TAGAACTGTC TGTGAACAGA GAGACCCCTG TGGGTCCATG CTAACAAAGA 1718  
 CAAAAGTCTG TCTTTCTCTGA ACCATCTGGA TAACCTTACA GAAATGGACT GGAGCTCATC 1778  
 TSCAAAAGGC CTCCTGTGAAA GACTGGTTTT CTGCCAATGA CCAACAGGCC AAGATTCTCC 1838  
 TCTTGTGATT TCTTTAAAAG AATGACTATA TAATTIATTT CCACTAAAAA TATTGTTCTC 1898  
 GCATTCATTT TATAGCAAC AACAAATGGT AAAACTCACT GTGATCAATA TTTTATATC 1958  
 ATGCAAAATA TGTTTAAAT AAAACGAAAA TGTATTAT 1997

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 419 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met His Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala  
 1 5 10 15  
 Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Phe  
 20 25 30  
 Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala  
 35 40 45  
 Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser  
 50 55 60

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Ser Val Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met  
 65 70 75 80  
 Tyr Lys Cys Glu Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln  
 85 90 95  
 Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala  
 100 105 110  
 His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys  
 115 120 125  
 Thr Glu Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe  
 130 135 140  
 Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr  
 145 150 155 160  
 Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr  
 165 170 175  
 Ser Thr Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu  
 180 185 190  
 Ser Glu Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser  
 195 200 205  
 Cys Arg Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile  
 210 215 220  
 Ile Arg Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn  
 225 230 235 240  
 Lys Thr Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys  
 245 250 255  
 Leu Ala Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser  
 260 265 270  
 Thr Asp Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu  
 275 280 285  
 Glu Thr Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys  
 290 295 300  
 Gly Pro His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys  
 305 310 315 320  
 Asn Lys Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu  
 325 330 335  
 Asn Thr Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro  
 340 345 350  
 Leu Asn Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys  
 355 360 365  
 Cys Leu Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr  
 370 375 380  
 Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser  
 385 390 395 400  
 Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro  
 405 410 415  
 Gln Met Ser

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr Asn Thr Glu Ile Leu  
 1 5 10 15

Lys

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1835 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
 (B) LOCATION: 158..1412

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGGCCGCGT CGAGCGAAAA GTTGCAGGCC GCCAGGTCCC GGGAGACGCT CGCCCAGGGG 60  
 GGTCCCCGGG AGGAACCCAC GGGACAGGGA CCAGGAGAGG ACCTTAGCCT CACGCCCCAG 120  
 CCTGGCCGAG CCAACGGACC GGCTCCCTG CTCCCGGTCC ATCCACC ATG CAC TTG 176  
 Met His Leu  
 CTG TGC TTC TTG TCT CTG GCG TGT TCC CTG CTC GCC SCT GCG CTG ATC 224  
 Leu Cys Phe Leu Ser Leu Ala Cys Ser Leu Leu Ala Ala Ala Leu Ile  
 5 10 15  
 CCC AGT CCG CGC GAG GCG CCC GCC ACC GTC GCC GCC TTC GAG TCG GSA 272  
 Pro Ser Pro Arg Glu Ala Pro Ala Thr Val Ala Phe Glu Ser Gly  
 20 25 30 35  
 CTG GGC TTC TCG GAA GCG GAG CCC GAC GGG GGC GAG CTC AAG GCT TTT 320  
 Leu Gly Phe Ser Glu Ala Glu Pro Asp Gly Gly Glu Val Lys Ala Phe  
 40 45 50  
 GAA GGC AAA GAC CTG GAG GAG CAG TTG CGG TCT GTG TCC AGC GTA GAT 368  
 Glu Gly Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser Val Asp  
 55 60 65  
 GAG CTG ATG TCT GTC CTG TAC CCA GAC TAC TGG AAA ATG TAC AAG TGC 416  
 Glu Leu Met Ser Val Leu Tyr Pro Asp Tyr Trp Lys Met Tyr Lys Cys  
 70 75 80  
 CAG CTG CGG AAA GGC GGC TGG CAG CAG CCC ACC CTC AAT ACC AGG ACA 464  
 Gln Leu Arg Lys Gly Gly Trp Gln Gln Pro Thr Leu Asn Thr Arg Thr  
 85 90 95  
 GGG GAC AGT GTA AAA TTT GCT GCT GCA CAT TAT AAC ACA GAG ATC CTG 512  
 Gly Asp Ser Val Lys Phe Ala Ala Ala His Tyr Asn Thr Glu Ile Leu  
 100 105 110 115  
 AAA AGT ATT GAT AAT GAG TGG AGA AAG ACT CAA TGC ATG CCA CGT GAG 560  
 Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln Cys Met Pro Arg Glu  
 120 125 130  
 GTG TGT ATA GAT GTG GGG AAG GAG TTT GGA GCA GCC ACA AAC ACC TTC 608  
 Val Cys Ile Asp Val Gly Lys Glu Phe Gly Ala Ala Thr Asn Thr Phe  
 135 140 145  
 TTT AAA CCT CCA TGT GTG TCC GTC TAC AGA TGT GGG GGT TGC TGC AAC 656  
 Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys Gly Gly Cys Cys Asn  
 150 155 160  
 AGC GAG GGG CTG CAG TGC ATG AAC ACC AGC ACA GGT TAC CTC AGC AAG 704  
 Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr Gly Tyr Leu Ser Lys  
 165 170 175  
 ACG TTG TTT GAA ATT ACA GTG CCT CTC TCA CAA GGC CCC AAA CCA GTC 752  
 Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln Gly Pro Lys Pro Val



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180	185	190	195	
ACA ATC AGT TTT GCC AAT CAC ACT TCC TGC CGG TGC ATG TCT AAA CTG Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg Cys Met Ser Lys Leu 200 205 210				800
GAT GTT TAC AGA CAA GTT CAT TCA ATT ATT AGA CGT TCT CTG CCA GCA Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg Arg Ser Leu Pro Ala 215 220 225				848
ACA TTA CCA CAG TGT CAG GCA GCT AAC AAG ACA TGT CCA ACA AAC TAT Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr Cys Pro Thr Asn Tyr 230 235 240				896
GTG TGG AAT AAC TAC ATG TGC CGA TGC CTG SCT CAG CAG GAT TTT ATC Val Trp Asn Asn Tyr Met Cys Arg Cys Leu Ala Gln Gln Asp Phe Ile 245 250 255				944
TTT TAT TCA AAT GTT GAA GAT GAC TCA ACC AAT GGA TTC CAT GAT GTC Phe Tyr Ser Asn Val Glu Asp Asp Ser Thr Asn Gly Phe His Asp Val 260 265 270 275				992
TGT GGA CCC AAC AAG GAG CTG GAT GAA GAC ACC TGT CAG TGT GTC TGC Cys Gly Pro Asn Lys Glu Leu Asp Glu Asp Thr Cys Gln Cys Val Cys 280 285 290				1040
AAG GGG GGG CTT CGG CCA TCT AGT TGT GGA CCC CAC AAA GAA CTA GAT Lys Gly Gly Leu Arg Pro Ser Ser Cys Gly Pro His Lys Glu Leu Asp 295 300 305				1088
AGA GAC TCA TGT CAG TGT CTC TGT AAA AAC AAA CTT TTC CCT AAT TCA Arg Asp Ser Cys Gln Cys Val Cys Lys Asn Lys Leu Phe Pro Asn Ser 310 315 320				1136
TGT GGA GCC AAC AGG GAA TTT GAT GAG AAT ACA TGT CAG TGT GTA TGT Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr Cys Gln Cys Val Cys 325 330 335				1184
AAA AGA ACG TGT CCA AGA AAT CAG CCC CTG AAT CCT GGG AAA TGT GCC Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn Pro Gly Lys Cys Ala 340 345 350 355				1232
TGT GAA TGT ACA GAA AAC ACA CAG AAG TGC TTC CTT AAA GGG AAG AAG Cys Glu Cys Thr Glu Asn Thr Gln Lys Cys Phe Leu Lys Gly Lys Lys 360 365 370				1280
TTC CAC CAT CAA ACA TGC AGT TGT TAC AGA AGA CCG TGT GCG AAT CCA Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg Pro Cys Ala Asn Arg 375 380 385				1328
CTG AAG CAT TGT GAT CCA GGA CTG TCC TTT AGT GAA GAA GTA TGC CGC Leu Lys His Cys Asp Pro Gly Leu Ser Phe Ser Glu Glu Val Cys Arg 390 395 400				1376
TGT GTC CCA TCG TAT TGG AAA AGG CCA CAT CTG AAC TAAGATCATA Cys Val Pro Ser Tyr Trp Lys Arg Pro His Leu Asn 405 410 415				1422
CCAGTCTTCA GTCACTCACA GTCACTTACT CTCTTGAAGA CTGTTGGAAC AGCACTTAGC ACTGCTATG CACAGAAAGA CTCTGTGGGA CCACATGGTA ACAGAGGCCC AAGTCTGTGT TTATGGAACC ATGTGGATTA CTGCGGGAGA GGACTGGCAC TCATGTGCAA AAAAAACCTC TTCARAGACT GGTCTTCTGC CAGGGACCAG ACAGCTGAGG TTTTCTCTT GTGATTTAA AAAAGAATGA CTATATAATT TATTCACACT AAAAATATG CTCTGCACTT CATTITTATA GCAATAACAA TTGTAAGAC TCACTGTGAT CAGTATTTT ATAACATGCA AAACATATGT TAAATAAAAA TGAAATGT ATTATAAAAA AAAAAAAAAA AAAAAAAAAA GCTT				1482 1542 1602 1662 1722 1782 1836

(2) INFORMATION FOR SEQ ID NO:11:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 415 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: protein.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met His Leu Leu Cys Phe Leu Ser Leu Ala Cys Ser Leu Leu Ala Ala  
 1 5 10 15  
 Ala Leu Ile Pro Ser Pro Arg Glu Ala Pro Ala Thr Val Ala Ala Phe  
 20 25 30  
 Glu Ser Gly Leu Gly Phe Ser Glu Ala Glu Pro Asp Gly Gly Glu Val  
 35 40 45  
 Lys Ala Phe Glu Gly Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser  
 50 55 60  
 Ser Val Asp Glu Leu Met Ser Val Leu Tyr Pro Asp Tyr Trp Lys Met  
 65 70 75 80  
 Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln Gln Pro Thr Leu Asn  
 85 90 95  
 Thr Arg Thr Gly Asp Ser Val Lys Phe Ala Ala Ala His Tyr Asn Thr  
 100 105 110  
 Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln Cys Met  
 115 120 125  
 Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Ala Ala Thr  
 130 135 140  
 Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys Gly Gly  
 145 150 155 160  
 Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr Gly Tyr  
 165 170 175  
 Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln Gly Pro  
 180 185 190  
 Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg Cys Met  
 195 200 205  
 Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg Arg Ser  
 210 215 220  
 Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr Cys Pro  
 225 230 235 240  
 Thr Asn Tyr Val Trp Asn Asn Tyr Met Cys Arg Cys Leu Ala Gln Gln  
 245 250 255  
 Asp Phe Ile Phe Tyr Ser Asn Val Glu Asp Asp Ser Thr Asn Gly Phe  
 260 265 270  
 His Asp Val Cys Gly Pro Asn Lys Glu Leu Asp Glu Asp Thr Cys Gln  
 275 280 285  
 Cys Val Cys Lys Gly Gly Leu Arg Pro Ser Ser Cys Gly Pro His Lys  
 290 295 300  
 Glu Leu Asp Arg Asp Ser Cys Gln Cys Val Cys Lys Asn Lys Leu Phe  
 305 310 315 320  
 Pro Asn Ser Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr Cys Gln  
 325 330 335  
 Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn Pro Gly  
 340 345 350  
 Lys Cys Ala Cys Glu Cys Thr Glu Asn Thr Gln Lys Cys Phe Leu Lys  
 355 360 365  
 Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg Pro Cys  
 370 375 380  
 Ala Asn Arg Leu Lys His Cys Asp Pro Gly Leu Ser Phe Ser Glu Glu  
 385 390 395 400

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Val Cys Arg Cys Val Phe Ser Tyr Trp Lys Arg Pro His Leu Asn  
405 410 415

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1741 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 453..1706

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCGCCCGCCG AGCGCTCCCG GCGGAGCCGC CGGGCCGCGG AGGGCGCGCT 60  
 GCGAGCGGCC ACTGGGTCCT GCTTCCCTCC TCTCTCTCCC TCTCTCTCCT CTTCTCTCTC 120  
 TCTCGCTTTT CCACCGCTCC CGAGCGAGCG CACGCTCGGA TGTCCGGTTT CTTGGTGGGT 180  
 TTTTACCTG SCARAGTCCG GATAACTTCG GTGAGAATT GCAAAGAGGC TGGGAGCTCC 240  
 CCTGAGGCG TCTGGAGCT GCTGCGCGCG TCGCATCTTC TCCATCCCGG GGATTCTACT 300  
 GCGTTGAGATA TTGCGAGGGG AGGGAGGGGG GTGAGGACAG CAAAAGAAA GGGGTGGGGG 360  
 GGGGAGAGAGA AAAGGAAAAG AAGGAGCCCTC GGAATTGTGC CCGCATTCCT GCGCTGCCCC 420  
 GCGGCCCCCC TCGCTCTGCG CATCTCCGCA CA ATG CAC TTG CTG GAG ATG CTC 473  
 Met His Leu Leu Glu Met Leu  
 1 5  
 TCC CTG GGC TGC TGC CTC GCT GCT GGC GCC GTG CTC CTG GGA CCC CGG 521  
 Ser Leu Gly Cys Cys Leu Ala Ala Gly Ala Val Leu Leu Gly Pro Arg  
 10 15 20  
 CAG CCG CCC GTC GCC GCC GCC TAC GAG TCC GGG CAC GGC TAC TAC GAG 569  
 Gln Pro Pro Val Ala Ala Ala Tyr Glu Ser Gly His Gly Tyr Tyr Glu  
 25 30 35  
 GAG GAG CCC GGT GCC GGG GAA CCC AAG GCT CAT GCA AGC AAA GAC CTG 617  
 Glu Glu Pro Gly Ala Gly Glu Pro Lys Ala His Ala Ser Lys Asp Leu  
 40 45 50 55  
 GAA GAG CAG TTS CGA TCT GTG TCC AGT GTG GAT GAA CTC ATG ACA GTA 665  
 Glu Glu Gln Leu Arg Ser Val Ser Ser Val Asp Glu Leu Met Thr Val  
 60 65 70  
 CTT TAC CCA GAA TAC TGG AAA ATG TTC AAA TGT CAG TTG AGG AAA GGA 713  
 Leu Tyr Pro Glu Tyr Trp Lys Met Phe Lys Cys Gln Leu Arg Lys Gly  
 75 80 85  
 GGT TGG CAA CAC AAC AGG GAA CAC TCC AGC TCT GAT ACA ASA TCA GAT 761  
 Gly Trp Gln His Asn Arg Glu His Ser Ser Ser Asp Thr Arg Ser Asp  
 90 95 100  
 GAT TCA TTG AAA TTT GCC GCA GCA CAT TAT AAT GCA GAG ATC CTG AAA 809  
 Asp Ser Leu Lys Phe Ala Ala Ala His Tyr Asn Ala Glu Ile Leu Lys  
 105 110 115  
 AST ATT GAT ACT CAA TGG AGA AAA ACC CAG GGC ATG CCA CGT GAA GTG 857  
 Ser Ile Asp Thr Glu Trp Arg Lys Thr Gln Gly Met Pro Arg Glu Val  
 120 125 130 135  
 TGT GTG GAT TTG GGG AAA GAG TTT GGA GCA ACT ACA AAC ACC TTC TTT 905  
 Cys Val Asp Leu Gly Lys Glu Phe Gly Ala Thr Thr Asn Thr Phe Phe  
 140 145 150  
 AAA CCC CCG TGT GTA TCC ATC TAC AGA TGT GGA GGT TGC TGC AAT AST 953  
 Lys Pro Pro Cys Val Ser Ile Tyr Arg Cys Gly Gly Cys Cys Asn Ser  
 155 160 165

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GAA GGA CTC CAG TGT ATG AAT ATC AGC ACA AAT TAC ATC AGC AAG ACA Glu Gly Leu Gln Cys Met Asn Ile Ser Thr Asn Tyr Ile Ser Lys Thr 170 175 180	1001
TTG TTT GAG ACT ACA GTG CCT CTC TCT CAT GGC CCC AAA CCT GTA ACA Leu Phe Glu Ile Thr Val Pro Leu Ser His Gly Pro Lys Pro Val Thr 185 190 195	1049
GTC AGT TTT GCC AAT CAC ACG TCC TGC CGA TGC ATG TCT AAG TTG GAT Val Ser Phe Ala Asn His Thr Ser Cys Arg Cys Met Ser Lys Leu Asp 200 205 210 215	1097
GTT TAC AGA CAA GTT CAT TCT ATC ATA AGA CGT TCC TTG CCA GCA ACA Val Tyr Arg Gln Val His Ser Ile Ile Arg Arg Ser Leu Pro Ala Thr 220 225 230	1145
CAA ACT CAG TGT CAT GTG GCA AAC AAG ACC TGT CCA AAA AAT CAT GTC Gln Thr Gln Cys His Val Ala Asn Lys Thr Cys Pro Lys Asn His Val 235 240 245	1193
CCG AAT AAT CAG ATT TGC AGA TGC TTA GCA CAG CAC GAT TTT CGT TTC Trp Asn Asn Gln Ile Cys Arg Cys Leu Ala Gln His Asp Phe Gly Phe 250 255 260	1241
TCT TCT CAC CTT GGA GAT TCT GAC ACA TCT GAA GGA TTC CAT ATT TGT Ser Ser His Leu Gly Asp Ser Asp Thr Ser Glu Gly Phe His Ile Cys 265 270 275	1289
GGG CCC AAC AAA GAG CTG GAT GAA GAA ACC TGT CAA TGC GTC TGC AAA Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr Cys Gln Cys Val Cys Lys 280 285 290 295	1337
GGA GGT GTG CGG CCC ATA AGC TGT GGC CCT CAC AAA GAA CTA GAC AGG Gly Gly Val Arg Pro Ile Ser Cys Gly Pro His Lys Glu Leu Asp Arg 300 305 310	1385
GCA TCA TGT CAG TGC ATG TGC AAA AAC AAA CTG CTC CCC AGT TCC TGT Ala Ser Cys Gln Cys Met Cys Lys Asn Lys Leu Leu Pro Ser Ser Cys 315 320 325	1433
GGG CCT AAC AAA GAA TTT GAT GAA GAA AAG TGC CAG TGT GTA TGT AAA Gly Pro Asn Lys Glu Phe Asp Glu Glu Lys Cys Gln Cys Val Cys Lys 330 335 340	1481
AAG ACC TGT CCC AAA CAT CAT CCA CTA AAT CCT GCA AAA TGC ATC TGC Lys Thr Cys Pro Lys His His Pro Leu Asn Pro Ala Lys Cys Ile Cys 345 350 355	1529
GAA TGT ACA GAA TCT CCC AAT AAA TGT TTC TTA AAA GGA AAA AGA TTT Glu Cys Thr Glu Ser Pro Asn Lys Cys Phe Leu Lys Gly Lys Arg Phe 360 365 370 375	1577
CAT CAC CAG ACA TGC AGT TGT TAC AGA CCA CCA TGT ACA GTC CGA ACG His His Gln Thr Cys Ser Cys Tyr Arg Pro Pro Cys Thr Val Arg Thr 380 385 390	1625
AAA CGC TGT GAT GCT GGA TTT CTG TTA GCT GAA GAA GTG TGC CGC TGT Lys Arg Cys Asp Ala Gly Phe Leu Leu Ala Glu Glu Val Cys Arg Cys 395 400 405	1673
GTA CGC ACA TCT TGG AAA AGA CCA CTT ATG AAT TAAGCGAAGA AAGCACTACT Val Arg Thr Ser Trp Lys Arg Pro Leu Met Asn 410 415	1726
CGCTATATAG TGTCG	1741

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 418 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met His Leu Leu Glu Met Leu Ser Leu Gly Cys Cys Leu Ala Ala Gly

-continued

1	5	10	15
Ala Val Leu Leu Gly Pro Arg Gln Pro Pro Val Ala Ala Ala Tyr Glu	20	25	30
Ser Gly His Gly Tyr Tyr Glu Glu Glu Pro Gly Ala Gly Glu Pro Lys	35	40	45
Ala His Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser Ser	50	55	60
Val Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met Phe	65	70	75
Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu His Ser	80	85	90
Ser Ser Asp Thr Arg Ser Asp Asp Ser Leu Lys Phe Ala Ala Ala His	95	100	105
Tyr Asn Ala Glu Ile Leu Lys Ser Ile Asp Thr Glu Trp Arg Lys Thr	110	115	120
Gln Gly Met Pro Arg Glu Val Cys Val Asp Leu Gly Lys Glu Phe Gly	125	130	135
Ala Thr Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Ile Tyr Arg	140	145	150
Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Ile Ser	155	160	165
Thr Asn Tyr Ile Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser	170	175	180
His Gly Pro Lys Pro Val Thr Val Ser Phe Ala Asn His Thr Ser Cys	185	190	195
Arg Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile	200	205	210
Arg Arg Ser Leu Pro Ala Thr Gln Thr Gln Cys His Val Ala Asn Lys	215	220	225
Thr Cys Pro Lys Asn His Val Trp Asn Asn Gln Ile Cys Arg Cys Leu	230	235	240
Ala Gln His Asp Phe Gly Phe Ser Ser His Leu Gly Asp Ser Asp Thr	245	250	255
Ser Glu Gly Phe His Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu	260	265	270
Thr Cys Gln Cys Val Cys Lys Gly Gly Val Arg Pro Ile Ser Cys Gly	275	280	285
Pro His Lys Glu Leu Asp Arg Ala Ser Cys Gln Cys Met Cys Lys Asn	290	295	300
Lys Leu Leu Pro Ser Ser Cys Gly Pro Asn Lys Glu Phe Asp Glu Glu	305	310	315
Lys Cys Gln Cys Val Cys Lys Lys Thr Cys Pro Lys His His Pro Leu	320	325	330
Asn Pro Ala Lys Cys Ile Cys Glu Cys Thr Glu Ser Pro Asn Lys Cys	335	340	345
Phe Leu Lys Gly Lys Arg Phe His His Gln Thr Cys Ser Cys Tyr Arg	350	355	360
Pro Pro Cys Thr Val Arg Thr Lys Arg Cys Asp Ala Gly Phe Leu Leu	365	370	375
Ala Glu Glu Val Cys Arg Cys Val Arg Thr Ser Trp Lys Arg Pro Leu	380	385	390
Met Asn	395	400	405

-continued

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 10 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ala Val Val Met Thr Gln Thr Pro Ala Ser  
 5 10

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 22 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCTCTCTGT GCTTGAGTTG AG

22

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 22 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TCTCTCTGT CCTGAGTTG AG

22

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 55 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGTGCTGCAG CAAATTTTAT AGTCTCTTCT GTGGCGGCGG CGGCAGCGGG CGCCTCGGA  
 GGACC

60

65

## (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 30 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTGGCAGGA ACTGCTAATA ATGGAATGAA

30

-continued

## (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 34 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGGCTCCGGC TCCGAGAGGT CGAGTCCGGA CTCGTGATGG TGATGGTGAT GGGCGGGGGC 60  
 GCGCGCGGGC GCCTCCGAG GACC 84

## (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 31 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTATTATAAT GTCTCCACC AAATTTATA G 31

## (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 93 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTTCGCTGCC TGACACTGTG GTAGTGTTC TCGCGGCCGC TAGTGATGGT GATGGTGATG 60  
 AATAATGGAA TGAAGTGTG TGTAACATC CAG 93

## (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 18 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CATGTACGAA CCGCCAGG 18

## (2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 20 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AATGACCAGA GAGAGGCGAG 20

-continued

## (2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GCCACG3TAG GTCGCGT

18

## (2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TTTCTTGAC AGGCTTAT

18

## (2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATCTGAAAA GTAAGTATGG G

21

## (2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ATGACTTGAC AGGTATGAT

20

## (2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AGCAAGACGG TGGGTATTGT

20

## (2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs



-continued

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCCTTCCTTG TAGTTATTG AA

22

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CCACAGTGAG TATGAATTAA

20

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TCTTCCTCAA SGTGTCAG

18

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GGAGATGGTA GCAGAAATG

18

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CTATTGTCT AGACTCAACA GAT

23

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: DNA (genomic)  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:  
 CAAACATGCA GGTAAGAGAT CC 22

(2) INFORMATION FOR SEQ ID NO:35:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA (genomic)  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:  
 TGTTCCTCTA GCTGTTACAG A 21

(2) INFORMATION FOR SEQ ID NO:36:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 24 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA (genomic)  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:  
 GGCGAGGTCA AGGTAGGTGC AAGG 24

(2) INFORMATION FOR SEQ ID NO:37:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 26 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA (genomic)  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:  
 ATTGTCTTTG ACAAGCTTTT TGAAGG 26

(2) INFORMATION FOR SEQ ID NO:38:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA (genomic)  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:  
 GAGATCCTGA AAAGTAAGTA G 21

(2) INFORMATION FOR SEQ ID NO:39:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 24 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA (genomic)  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

-continued

TTTGACTCGA CAGGATTGA TAAT

24

(2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CTCAGCAAGA CGGTAGGTAT

20

(2) INFORMATION FOR SEQ ID NO:41:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TTGTCCCTTG TAGTGTGTG AAATT

25

(2) INFORMATION FOR SEQ ID NO:42:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

ACATTACCAC AGTGAGTATG

20

(2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GTCTCCCCAA AAGGTGTCAG GCAGCT

26

(2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

AATGTTGAAG ATGTAAGTA AAA

23

(2) INFORMATION FOR SEQ ID NO:45:

-continued

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 16 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

TCTAGACTCA ACCAAT

16

(2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 22 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CAAACATGCA GGTAAGGAST GT

22

(2) INFORMATION FOR SEQ ID NO:47:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 24 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TTTCCCTA GTGTACAG AAGA

24

(2) INFORMATION FOR SEQ ID NO:48:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 2991 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GTTTAAAGTA GAGACGGGGT TTCACCAACG GTTGAATAA TTTATCATGG TCTCCCTAAG 60  
 ATGACGCGTG TTAGCTAGGA TGGTCTGGAT CTCCTGACCT CATGATCCAC CCGCCTCGGC 120  
 CTCCTCAAGT GCTGGGATTA CAGGCGTGAG CCACCGTGTC CGACCAACCT TAAGACAAAC 180  
 AACTACTGCA TGATGTGTTT TGGAGACCTT TTTTATTTC AAATRAATTT TTGCCASCAT 240  
 TTTCTGACTC AAAGTATAGC AGCAGGAAGA TAACACTTTT GTGAGAAAAA AGTTTGAATA 300  
 CAGCTTACTG CTGTATTAA ATGAACAGT AGTAAATATG ATATTAATAT ATTTTGGATA 360  
 TATTTGAGT TGTGTGATT TCCAGTCTTC ACCCGCTGCT AGGCGTGTGG GTGTTGAAA 420  
 TGCCGTGTTT TCTCAATTT GTTGTCTAT TAGAATCCTG ATGTCCAAGC CTTACTCCAG 480  
 TTAGACCAGT TAAGCCAGAA AGCAGGAAGG TGTACTCAAG CATCTGTTTT TTCAAAATCT 540  
 CCTTTGTGA TGCCAAAGTC AATCAAAGT TAGAATCATT GTAATAGCAA ATGGTTGAAT 600  
 GGAACTCCA CTTCTATTTC AATCCTACC CCAGTCTGCC CTTAGCTGTT CTCTTTCAC 660  
 AGATCTATCA ATGTCTGAAG ATAACCTAGG CAGGCTGATC AAATATGCAT AGAGCAGGAA 720

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GACAGCAAGA GAGTGATACA CTGACCATGT TCCAAATCAC AAAACATCTC AACAGGCTAG	780
ATCATGGACC GAGTCTGATG GAGTGGAAIT TCATAAGAT ACATAAAAA GCATCTTGGA	840
TACAGTAAAC TTAATCCAC AAATACAGGG GAATTTAGAC GTGACTAAGT AGCAGTACAT	900
ATGAAAAATT ATTSAGGAAT TTGTGTGACT TTAAGGGTAG TGTGAGTCAA CACTGTSATT	960
TGGCTGCCAG AAAATAAAT CAATCCAGG CTGTATCAAC AAAGSCATAC TGTCCATTCT	1020
GCATGCTCAT TACAGCACTA AGTACCGAGC CAIGTTCTCA ACCGCATACT TCATGAACAT	1080
GGAAAGCTAA CAGTATGGTT AAGGGGGGAA ACTGGAACTG TCATCTTGGG GAATAAAGG	1140
GATATTAGC CAGSAGTAA GTTAGCTTAG GGAGACCATG ATAAATATTT TCAAAATATT	1200
TGAAGGACTC AGTTGTGGAA GTGAGATTAG ATTATTTGT TAAACTTCCA GGAGTCAAAA	1260
GCAATAGAGA GATAGAAGGA AATGCTTTTC AGCAGTGTG CTCATCAATA AAGGGAGTGA	1320
ACAGCCACAC AGAATGGAAG GTTCCCTGTC CTTTGAGATA TTAAAGCCTT CAAGTAAATT	1380
ATGGGTGAGG AGTTTCAANT CTAGAGTTGA ACCAGATAAG AAAGTCTCTT CTTCGGTAA	1440
GATATTATGG ACCTATAACA TCTGTGTACT TAAAGTAGA TTGGGAGTGA AAGGCAGACT	1500
TTTATGTTC TGTACACTGT TGAACCCCT TAGCGTGGTC CTCGTAAACC TGCTACCCCT	1560
GCCCCAAGGA GGCAGCTAGC CAATGCCACC AGCCCAACGG AAACCCAGT GCTTTTCCAA	1620
TGGGGAAATG CAGTCACTTT TCTTSGATG CTACACATCC TTCTGGAAT ATGTCTCACA	1680
CACATCTCTC TTATACACC CTTTTTCAA GTAAACCAAC TTCTTGAGA AGCTGACAA	1740
GTGTCTTTT ACTTCCACG AAGATTCTGG CCCTTCTCTT CACCTGTAG AAGTTAGGA	1800
TTCCAAAGGG ATCATTAGCA TCCATCCCAA CAGCCTGCAC TGCATCCTGA GAAGTGGGT	1860
TTTGGATCA TCAGGCAACT TTCAACTACA CAGACCAAGG GAGAGAGGGG ACCCCTCCGA	1920
GCTCCCATAG GGTCTCTGA CATAGTGATG ACCTTTTCC AAACTTGAG CAGGGCCTG	1980
GGGGCCAGGC GTGGGGAGG GAGGACAAGA ACTCGGGAGT GGGCAGCAT AAAGCGGGG	2040
CTCCCTCAC CCCAGGTGC CAGTTTCTC CCCGCTGCAC GTGGTCCAGG GTGGTCCAT	2100
CACCTCTAAA GCGGTCCCG CCAACCGCCA GCCCCGGGAC TGAACTTGCC CCTCCGCCG	2160
CCCGTCCCC GCAAGGACA GGGGGGGGA GGGAGAGATC CAGAGGGGG CTGGGGGAGG	2220
TGGGCGGCC GGGAGGAGG CGAGGGAAC GGGGAGCTCC AGGGAGAGCG CTTCGAGGG	2280
AGAGTGAGAG GGGAGGGCAG CCGGGCTCG GCACGCTCC TCCCTCGGC GCTTCTCTC	2340
ACATAAGCGC AGGCAGAGG CGCGTCAGTC ATGCCCTGCC CCTGCGCCC CCGCCGCCG	2400
CSCCGCCGT CAGCCCGGG CGCTCTGGAG GATCCTGCC CGCGCGCTC CCGGGCCCCG	2460
CCGCGCCAG CCGCCCCGC GGGCTCTCT CCGCCCCCG CACCGCCGC AGCGCCCCG	2520
CCGAGCGCC GCGCGCCCG CTCTCTCAC TTCGGGAAG GGGAGGAGG AGGGGGACGA	2580
GGGCTCTGGC GGGTTTGGG GGGCTGAACA TCGCGGGTG TTCTGGTGT CCGCGCCCCG	2640
CCTCTCCAAA AAGTACACC GAGCGGACC GCGCGGGGT CCTCCTCGC CCTCGCTCA	2700
CCTCGCGGG TCCGAATGC GGGAGCTCG ATGTCCGTT TCCTGTGAGG CTTTACCTG	2760
ACACCCSCCG CCTTCCCCG GCACTGGTG GAGGGGCGC CTGCAAGTT GGAACGCGG	2820
AGCCCCGAC CCGTCCCCG CGCTCCGGC TCGCCAGGG GGGGTGCGG GAGGAGCCC	2880
GGGGAGAGG GACAGGAGG GGGCCSCGG CTCGAGGGG GCGCGCGCC CCCACCCCTG	2940
CCCCCGCAG CGGACCGGT CCCCACCCC GGTCTTCCA CCATGCATT G	2991

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- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 20 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:  
 CACGGCTTAT GCAAGCAAAG 20
- (2) INFORMATION FOR SEQ ID NO:50:
- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 20 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:  
 AACACAGTTT TCCATAATAG 20
- (2) INFORMATION FOR SEQ ID NO:51:
- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 19 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:  
 Leu Ser Lys Thr Val Ser Gly Ser Glu Gln Asp Leu Pro His Glu Leu  
 1 5 10 15  
 His Val Glu
- (2) INFORMATION FOR SEQ ID NO:52:
- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 25 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:  
 GACGGACACA GATGGAGGTT TAAAG 25
- (2) INFORMATION FOR SEQ ID NO:53:
- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 196 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:  
 Met Arg Thr Leu Ala Cys Leu Leu Leu Leu Gly Cys Gly Tyr Leu Ala  
 1 5 10 15  
 His Val Leu Ala Glu Glu Ala Glu Ile Pro Arg Glu Val Ile Glu Arg  
 20 25 30

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Leu Ala Arg Ser Gln Ile His Ser Ile Arg Asp Leu Gln Arg Leu Leu  
 35 40 45  
 Glu Ile Asp Ser Val Gly Ser Glu Asp Ser Leu Asp Thr Ser Leu Arg  
 50 55 60  
 Ala His Gly Val His Ala Thr Lys His Val Pro Glu Lys Arg Pro Leu  
 65 70 75 80  
 Pro Ile Arg Arg Lys Arg Ser Ile Glu Glu Ala Val Pro Ala Val Cys  
 85 90 95  
 Lys Thr Arg Thr Val Ile Tyr Glu Ile Pro Arg Ser Gln Val Asp Pro  
 100 105 110  
 Thr Ser Ala Asn Phe Leu Ile Trp Pro Pro Cys Val Glu Val Lys Arg  
 115 120 125  
 Cys Thr Gly Cys Cys Asn Thr Ser Ser Val Lys Cys Gln Pro Ser Arg  
 130 135 140  
 Val His His Arg Ser Val Lys Val Ala Lys Val Glu Tyr Val Arg Lys  
 145 150 155 160  
 Lys Pro Lys Leu Lys Glu Val Gln Val Arg Leu Glu Glu His Leu Glu  
 165 170 175  
 Cys Ala Cys Ala Thr Thr Ser Leu Asn Pro Asp Tyr Arg Glu Glu Asp  
 180 185 190  
 Thr Asp Val Arg  
 195

## (2) INFORMATION FOR SEQ ID NO:54:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 241 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Met Asn Arg Cys Trp Ala Leu Phe Leu Ser Leu Cys Cys Tyr Leu Arg  
 5 10 15  
 Leu Val Ser Ala Glu Gly Asp Pro Ile Pro Glu Glu Leu Tyr Glu Met  
 20 25 30  
 Leu Ser Asp His Ser Ile Arg Ser Phe Asp Asp Leu Gln Arg Leu Leu  
 35 40 45  
 His Gly Asp Pro Gly Glu Glu Asp Gly Ala Glu Leu Asp Leu Asn Met  
 50 55 60  
 Thr Arg Ser His Ser Gly Gly Glu Leu Glu Ser Leu Ala Arg Gly Arg  
 65 70 75 80  
 Arg Ser Leu Gly Ser Leu Thr Ile Ala Glu Pro Ala Met Ile Ala Glu  
 85 90 95  
 Cys Lys Thr Arg Thr Glu Val Phe Glu Ile Ser Arg Arg Leu Ile Asp  
 100 105 110  
 Arg Thr Asn Ala Asn Phe Leu Val Trp Pro Pro Cys Val Glu Val Gln  
 115 120 125  
 Arg Cys Ser Gly Cys Cys Asn Asn Arg Asn Val Gln Cys Arg Pro Thr  
 130 135 140  
 Gln Val Gln Leu Arg Pro Val Gln Val Arg Lys Ile Glu Ile Val Arg  
 145 150 155 160  
 Lys Lys Pro Ile Phe Lys Lys Ala Thr Val Thr Leu Glu Asp His Leu  
 165 170 175

-continued

Ala Cys Lys Cys Glu Thr Val Ala Ala Ala Arg Pro Val Thr Arg Ser  
 180 185 190  
 Pro Gly Gly Ser Gln Glu Gln Arg Ala Lys Thr Pro Gln Thr Arg Val  
 195 200 205  
 Thr Ile Arg Thr Val Arg Val Arg Arg Pro Pro Lys Gly Lys His Arg  
 210 215 220  
 Lys Phe Lys His Thr His Asp Lys Thr Ala Leu Lys Glu Thr Leu Gly  
 225 230 235 240  
 Ala

## (2) INFORMATION FOR SEQ ID NO:55:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 149 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Ala Gly  
 1 5 10 15  
 Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser Ala Gly  
 20 25 30  
 Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Gln Glu Val Trp Gly  
 35 40 45  
 Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp Val Val Ser Glu  
 50 55 60  
 Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro Ser Cys Val Ser Leu  
 65 70 75 80  
 Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu Asn Leu His Cys Val Pro  
 85 90 95  
 Val Glu Thr Ala Asn Val Thr Met Gln Leu Leu Lys Ile Arg Ser Gly  
 100 105 110  
 Asp Arg Pro Ser Tyr Val Glu Leu Thr Phe Ser Gln His Val Arg Cys  
 115 120 125  
 Glu Cys Arg Pro Leu Arg Glu Lys Met Lys Pro Glu Arg Cys Gly Asp  
 130 135 140  
 Ala Val Pro Arg Arg  
 145

## (2) INFORMATION FOR SEQ ID NO:56:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 191 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu  
 1 5 10 15  
 Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly  
 20 25 30  
 Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln  
 35 40 45



-continued

Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu  
50 55 60  
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu  
65 70 75 80  
Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro  
85 90 95  
Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His  
100 105 110  
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys  
115 120 125  
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Asn Pro Cys Gly  
130 135 140  
Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp Pro Gln Thr  
145 150 155 160  
Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln  
165 170 175  
Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg  
180 185 190

## (2) INFORMATION FOR SEQ ID NO:57:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 188 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Met Ser Pro Leu Leu Arg Arg Leu Leu Ala Ala Leu Leu Gln Leu  
5 10 15  
Ala Pro Ala Gln Ala Pro Val Ser Gln Pro Asp Ala Pro Gly His Gln  
20 25 30  
Arg Lys Val Val Ser Trp Ile Asp Val Tyr Thr Arg Ala Thr Cys Gln  
35 40 45  
Pro Arg Glu Val Val Val Pro Leu Thr Val Glu Leu Met Gly Thr Val  
50 55 60  
Ala Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly  
65 70 75 80  
Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln  
85 90 95  
Val Arg Met Gln Ile Leu Met Ile Arg Tyr Pro Ser Ser Gln Leu Gly  
100 105 110  
Glu Met Ser Leu Glu Gln His Ser Gln Cys Glu Cys Arg Pro Lys Lys  
115 120 125  
Lys Asp Ser Ala Val Lys Pro Asp Ser Pro Arg Pro Leu Cys Pro Arg  
130 135 140  
Cys Thr Gln His His Gln Arg Pro Asp Pro Arg Thr Cys Arg Cys Arg  
145 150 155 160  
Cys Arg Arg Arg Ser Phe Leu Arg Cys Gln Gly Arg Gly Leu Glu Leu  
165 170 175  
Asn Pro Asp Thr Cys Arg Cys Arg Lys Leu Arg Arg  
180 185

What is claimed is:

1. A purified and isolated VEGF-CAC<sub>156</sub> polypeptide that binds to human Flt4 receptor tyrosine kinase (VEGFR-3) and fails to bind to human KDR receptor tyrosine kinase (VEGFR-2), said polypeptide having an amino acid sequence comprising a portion of SEQ ID NO: 8 effective to permit binding to VEGFR-3, wherein the cysteine residue at position 156 of SEQ ID NO: 8 has been deleted or replaced by another amino acid.

2. A polypeptide according to claim 1 wherein said portion of SEQ ID NO: 8 is selected from the group consisting of:

(a) continuous portion having as its amino terminal residue an amino acid between residues 102 and 114 of SEQ ID NO: 8 and having as its carboxy terminal residue an amino acid between residues 212 and 228 of SEQ ID NO: 8, wherein the cysteine residue at position 156 of SEQ ID NO: 8 has been deleted or replaced by another amino acid;

(b) continuous portions that comprise an amino-terminal truncation of (a); and  
continuous portions that comprise a carboxy-terminal truncation of (a) or (b).

3. A polypeptide according to claim 2, wherein said polypeptide is capable of stimulating tyrosine phosphorylation of VEGFR-3 in a host cell expressing VEGFR-3.

4. A VEGF-C  $\Delta$ C<sub>156</sub> polypeptide according to claim 1 comprising amino acids 113 to 213 of SEQ ID NO: 8, wherein the cysteine residue at position 156 of SEQ ID NO: 8 has been deleted or replaced.

5. A VEGF-C  $\Delta$ C<sub>156</sub> polypeptide according to claim 1 comprising a continuous portion of SEQ ID NO: 8, said portion having as its amino terminal residue an amino acid between residues 102 and 114 of SEQ ID NO: 8, and having as its carboxy terminal residue an amino acid between residues 212 and 228 of SEQ ID NO: 8, wherein the cysteine residue at position 156 of SEQ ID NO: 8 has been deleted or replaced.

6. A VEGF-C  $\Delta$ C<sub>156</sub> polypeptide according to claim 1 wherein the cysteine residue at position 156 of SEQ ID NO: 8 has been replaced by a serine residue.

7. A composition comprising a polypeptide according to claim 1 in a pharmaceutically-acceptable diluent, adjuvant, excipient, or carrier.

8. A purified and isolated polypeptide multimer, wherein at least one monomer thereof is a polypeptide according to claim 1, and wherein said multimer is capable of binding to VEGFR-3.

9. A dimer according to claim 1.

10. A polypeptide according to claim 1 comprising amino acids 1 to 419 of SEQ ID NO: 8, wherein the cysteine residue at position 156 of SEQ ID NO: 8 has been deleted or replaced by another amino acid.

11. A method of imaging Flt4 receptor tyrosine kinase (VEGFR-3) in tissue suspected of containing VEGFR-3, comprising the steps of contacting tissue suspected of containing VEGFR-3 with a polypeptide of claim 1 and imaging VEGFR-3 in the tissue by detecting the polypeptide bound to the tissue.

12. A purified and isolated nucleic acid comprising a nucleotide sequence encoding a VEGF-C  $\Delta$ C<sub>156</sub> polypeptide that binds to human Flt4 receptor tyrosine kinase (VEGFR-3) and fails to bind to human KDR receptor tyrosine kinase (VEGFR-2), said polypeptide, having an amino acid sequence comprising a portion of SEQ ID NO: 8 effective to permit binding to VEGFR-3, wherein the cysteine residue at position 156 of SEQ ID NO: 8 has been deleted or replaced by another amino acid.

13. A vector comprising a nucleic acid according to claim 12.

14. A host cell transformed or transfected with a nucleic acid according to claim 12.

15. A method of making a polypeptide that binds to VEGFR-3, said method comprising the steps of:

(a) expressing a nucleic acid according to claim 12 in a host cell; and

(b) purifying a polypeptide that binds to VEGFR-3 from said host cell or from a growth medium of said host cell.

16. A polypeptide that binds to the extracellular domain of human Flt4 receptor tyrosine kinase (VEGFR-3),

said polypeptide comprising a fragment of a vertebrate prepro-VEGF-C amino acid sequence that binds to human VEGFR3, with the proviso that, in said polypeptide a conserved cysteine of the vertebrate prepro-VEGF-C has been deleted or replaced by another amino acid,

wherein the vertebrate prepro-VEGF-C amino acid sequence comprises an amino acid sequence that is encoded by a DNA of vertebrate origin which hybridizes to a non-coding strand complementary to nucleotides 352 to 1611 of SEQ ID NO: 7 under the following hybridization conditions: hybridization at 42° C. in a hybridization solution comprising 50% formamide, 5xSSC, 20 mM Na<sub>2</sub>PO<sub>4</sub>, pH 6.8; and washing in 0.2xSSC at 55° C.,

wherein nucleotides 352 to 1611 of SEQ ID NO: 7 encode a human prepro-VEGF-C having the amino acid sequence set forth in SEQ ID NO: 8 that is characterized by eight cysteine residues at positions 131, 156, 162, 165, 166, 173, 209, and 211 of SEQ ID NO: 8 that are conserved in human vascular endothelial growth factor (VEGF), human platelet derived growth factors A and B (PDGF-A, PDGF-B), human placenta growth factor (PIGF-1), and human vascular endothelial growth factor B (VEGF-B), and

wherein the conserved cysteine that has been deleted or replaced corresponds to position 156 of SEQ ID NO: 8.

17. A purified polypeptide according to claim 16 wherein the vertebrate is a human.

18. A polypeptide according to claim 16 wherein the vertebrate is a mouse.

19. A polypeptide according to claim 16 that binds VEGFR-3 and has reduced VEGFR-2 binding affinity relative to human VEGF-C having an amino acid sequence consisting of amino acids 103-227 of SEQ ID NO: 8.

20. A composition comprising a polypeptide according to claim 16 in a pharmaceutically-acceptable diluent, adjuvant, excipient, or carrier.

21. A polypeptide according to claim 16, wherein said polypeptide comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO: 11, wherein the cysteine residue at position 152 of SEQ ID NO: 11 has been deleted or replaced by another amino acid;

(b) amino-terminal truncations of (a); and

(c) carboxyl-terminal truncations of (a) or (b).

22. A polypeptide according to claim 16, wherein said polypeptide comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO: 13, wherein the cysteine residue at position 155 of SEQ ID NO: 13 has been deleted or replaced by another amino acid;

(b) amino-terminal truncations of (a); and

(c) carboxyl-terminal truncations of (a) or (b).

23. A purified and isolated nucleic acid comprising a nucleotide sequence encoding a polypeptide that binds to the extracellular domain of human Flt4 receptor tyrosine kinase (VEGFR-3),

said polypeptide comprising a fragment of a vertebrate prepro-VEGF-C amino acid sequence that binds to human VEGFR-3, with the proviso that, in said polypeptide, a conserved cysteine of the vertebrate prepro-VEGF-C has been deleted or replaced by another amino acid,

wherein the vertebrate prepro-VEGF-C amino acid sequence comprises an amino acid sequence that is encoded by a DNA of vertebrate origin which hybridizes to a non-coding strand complementary to nucleotides at 352 to 1611 of SEQ ID NO: 7 under the following hybridization condition: hybridization at 42° C. in a hybridization solution comprising 50% formamide, 5xSSC 20 mM Na<sub>2</sub>PO<sub>4</sub>, pH 6.8; and washing in 0.2xSSC at 55° C.,

wherein nucleotides 352 to 1611 SEQ ID NO: 7 encoded a human prepro-VEGF-C having the amino acid sequence set forth in SEQ ID NO: 8 that is characterized by eight cysteine residues at position 131, 156, 162, 165, 166, 173, 209, and 211 of SEQ ID NO: 8 that are conserved in human vascular endothelial growth factor (VEGF), human platelet derived growth factors A, and B (PDGF-A, PDGF-B), human placenta growth factor (PIGE-1), and human vascular endothelial growth factor B (VEGR-B), and

wherein the conserved cysteine that has been deleted or replaced corresponds to position 156 of SEQ ID NO: 8.

24. A vector comprising a nucleic acid according to claim 23.

25. A host cell transformed or transfected with a nucleic acid according to claim 23.

26. A method of making a polypeptide that binds to VEGFR-3, said method comprising the steps of:

(a) expressing a nucleic acid according to claim 23 in a host cell; and

(b) purifying a polypeptide that binds to VEGFR-3 from said host cell or from a growth medium of said host cell.

27. A polypeptide that binds to human Flt4 receptor tyrosine kinase (VEGFR-3), said polypeptide comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO: 8, wherein the cysteine residue at position 156 of SEQ ID NO: 8 has been deleted replaced by another amino acid;

(b) the amino acid sequence of SEQ ID NO: 11, wherein the cysteine residue at position 152 of SEQ ID NO: 11 has been deleted or replaced by another amino acid;

(c) the amino acid sequence of SEQ ID NO: 13, wherein the cysteine residue at position 155 of SEQ ID NO: 13 has been deleted or replaced by another amino acid;

(d) amino-terminal truncations of (a), (b), or (c); and

(e) carboxyl-terminal truncations of (a), (b), (c), or (d).

28. A polynucleotide comprising a nucleotide sequence that encodes a polypeptide that binds to human Flt4 receptor tyrosine kinase (VEGFR-3), said polypeptide comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO: 8, wherein the cysteine residue at position 156 of SEQ ID NO: 8 has been deleted or replaced by another amino acid;

(b) the amino acid sequence of SEQ ID NO: 11, wherein the cysteine residue at position 152 of SEQ ID NO: 11 has been deleted or replaced by another amino acid;

(c) the amino acid sequence of SEQ ID NO: 13, wherein the cysteine residue at position 155 of SEQ ID NO: 13 has been deleted or replaced by another amino acid;

(d) amino-terminal truncations of (a), (b), or (c); and

(e) carboxyl-terminal truncations of (a), (b), (c), or (d).

29. A polynucleotide according to claim 28, wherein the nucleotide sequence that encodes the peptide comprises a sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in SEQ ID NO: 7 from nucleotide 352 to 1611, wherein the cysteine codon at positions 817-819 has been deleted or replaced by a codon for an amino acid other than cysteine;

(b) the nucleotide sequence set forth in SEQ ID NO: 10 from nucleotide 168 to 1412, wherein the cysteine codon at positions 621-623 has been deleted or replaced by a codon for an amino acid other than cysteine;

(c) the nucleotide sequence set forth in SEQ ID NO: 12 from nucleotide 453 to 1706, wherein the cysteine codon at positions 915-917 has been deleted or replaced by a codon for an amino acid other than cysteine;

(d) 5'-deletion fragments of (a), (b), or (c); and

(e) 3'-deletion fragments of (a), (b), (c), or (d).

\* \* \* \* \*

COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

**IN THE MATTER OF:** Australian

Patent Application 696764

(73941/94). In the name of:

Human Genome Sciences Inc.

- and -

**IN THE MATTER OF:** Opposition

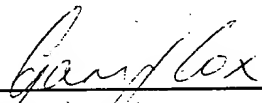
thereto by Ludwig Institute for Cancer

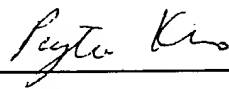
Research, under Section 59 of the

Patents Act.

**Annexure GBC-13**

This is **Annexure GBC-13** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
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**Gary Baxter Cox**

WITNESS:   
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Patent Attorney **PEYTEE KUO**

# Clinical Frontiers

## Human gene therapy

BARRY R. GOLDSPIEL, LAURENCE GREEN, AND KARIM ANTON CALIS

**Abstract:** Current concepts in gene transfer and its application to the treatment of human genetic disorders, cancer, and other diseases are discussed.

Gene therapy is a technique in which a functioning gene is inserted into a human cell to correct a genetic error or to introduce a new function to the cell. Many methods, including retroviral vectors, have been developed for ex vivo and in vivo gene insertion into cells. Some pharmacists have likened gene therapy to a sophisticated form of drug delivery and have envisioned an active role for the pharmacy profession. There are several safety and ethical issues related to manipulating the human genome that need to be understood. Current gene therapy efforts focus on gene insertion into somatic (non-

germinal) cells only.

Gene therapy has the potential to revolutionize the treatment of genetic disorders, diseases associated with a genetic component (e.g., cystic fibrosis), cancer, AIDS, and many other diseases. Gene transfer may also be used to better understand the biology of disease processes, such as the source of relapse in bone marrow transplant patients. The human genome project will undoubtedly lead to the identification, characterization, and understanding of genes that are responsible for many human diseases, and gene therapy trials are sure to expand accordingly.

To date, over 40 clinical trials have been approved and more than 110 patients have been entered in gene therapy studies. There are still

many technical obstacles to overcome before gene therapy can have widespread application. Injectable vectors need to be developed to simplify foreign gene administration. Perhaps the biggest problem to overcome will be engineering the target cells to be able to regulate gene expression according to physiologic needs.

Pharmacists should become knowledgeable about gene transfer techniques and possible clinical applications of gene therapy to keep abreast of the newest trends in medicine.

**Index terms:** Gene therapy; Genetic engineering; Neoplasms; Research; Transfection

Clin Pharm. 1993; 12:488-505

Recent advances in molecular biology and related disciplines have contributed to major developments in the diagnosis and treatment of human disease. Numerous diagnostic products and biological therapies have been produced by recombinant DNA technology. Identification and cloning of genes involved in various human diseases have heralded the era of human gene therapy, a technique in which a functioning gene is inserted into a human cell to correct a genetic error or to introduce a new function to the cell.<sup>1</sup> Viruses such as murine retroviruses, adenoviruses, herpes viruses, and parvoviruses are gaining wide use for introducing foreign genes into human cells.<sup>1,2</sup> The ultimate success and utility of human gene therapy depend in large part on the ability of scientists

to elucidate the structure, function, and regulation of human genes.<sup>3</sup> The human genome project, an international effort to map and sequence the entire genomes of man and several model organisms, will undoubtedly lead to the identification, characterization, and understanding of genes that are responsible for many human diseases.<sup>4</sup>

Gene therapy has the potential to revolutionize the treatment of genetic disorders, diseases associated with a genetic component (e.g., cystic fibrosis), cancer, AIDS, and many other diseases. Gene transfer may also be used to better understand the biology of disease processes. Some pharmacists have likened gene therapy to a sophisticated form of drug delivery and

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Kenneth Culver, M.D., Cellular Immunology Section, Metabolism Branch, National Cancer Institute, is acknowledged for his expert manuscript review.

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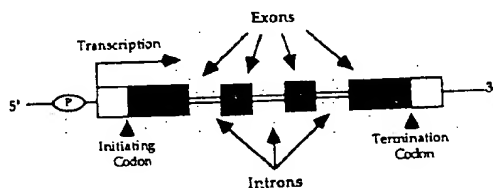
have envisioned an active role for the pharmacy profession.<sup>5</sup> Pharmacists, however, must understand the fundamentals of molecular biology if they are to keep pace with the rapid advances in gene therapy. This knowledge, integrated with expertise in pharmacotherapy, will allow pharmacists to contribute to the development of gene therapy trials and the care of patients undergoing this form of treatment. This article will review basic mechanics of gene transfer and gene therapy, describe the potential applications of gene transfer and gene therapy to better understanding of disease biology, and summarize the ongoing clinical trials employing gene transfer and gene therapy techniques.

### Gene Structure, Function, and Regulation

A gene is a segment of DNA with a unique order of purine and pyrimidine bases that encode a specific protein (Figure 1). It is estimated that the human genome contains 3 billion DNA base pairs or the equivalent of 50,000–100,000 genes divided among the chromosomes and the mitochondria.<sup>4</sup> Most genes from eukaryotic cells contain coding sequences (exons) that are interrupted by one or more noncoding regions (introns).<sup>6</sup> The regions adjacent to the introns and exons contain the initiation and termination sequences necessary to regulate messenger RNA (mRNA) transcription. Several similar sequences are found in the 5' regions of many different genes, possibly indicating that these sequences play an important role in gene regulation.<sup>6</sup>

The flow of information from gene to protein product involves many complicated, highly coordinated processes (Figure 2). The template for protein synthesis, mRNA, is formed in the nucleus as a complemen-

**Figure 1.** Basic gene structure. A gene is a sequence of chromosomal DNA required to produce a functional RNA or polypeptide product. The basic gene structure consists of both the actual coding sequences (exons) and sequences necessary for proper gene expression. The promoter region (P), located at the 5' terminus, includes the start codon and the sequences that initiate messenger RNA (mRNA) transcription. Very few genes exist as continuous coding sequences; most are interrupted by noncoding sequences (introns). Transcription of the exon regions determines the resultant amino acid sequence. Introns are transcribed in the nucleus to RNA but are not part of the cytoplasmic mRNA and therefore not part of the final protein product. Beyond the stop codon, an untranslated region at the 3' terminus is responsible for adding multiple polyadenosine residues to the mRNA.



*Reviews in the Clinical Frontiers section summarize recent research advances, such as those that contribute to an understanding of a disease process or the mechanism of drug action, describe new technologies or medical procedures that are likely to affect drug therapy, or describe likely new modes of drug therapy. Preference is given to articles that assess directions of ongoing research and critically evaluate therapeutic implications in new or rapidly changing areas.*

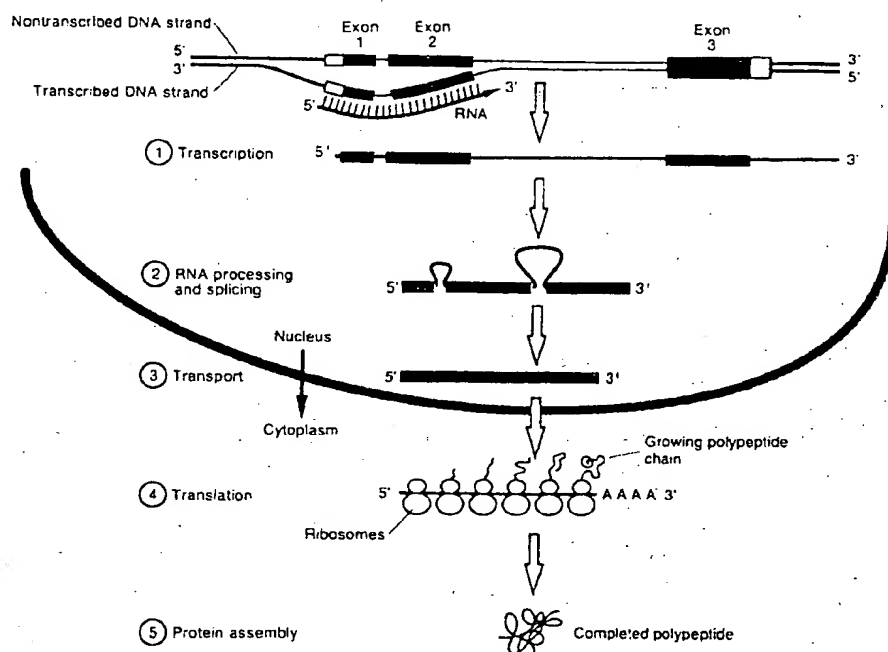
tary copy of a single-strand portion of DNA through a process called transcription. The mRNA base sequence is then decoded to form a protein through a process known as translation. Ribosomal RNA is responsible for protein synthesis under the direction of transfer RNA, which provides the molecular link between the coded sequence of RNA and that of the protein.<sup>6</sup>

The genetic code is composed of 64 codons, which are sequences of three adjacent bases that specify a particular amino acid. Although up to three different codons can specify the same amino acid, the genetic code is considered universal because all organisms use the same codons. This fact explains why a simple bacterium can translate a human gene into a polypeptide. Moreover, the DNA or RNA base sequence determines the amino acid sequence, and knowledge of the amino acid sequence can be used to deduce the possible DNA sequence that codes for the protein.<sup>7</sup>

The complex regulation of end-product protein production (gene expression) is controlled by many factors, including gene structure, transcription, translation, gene product amount (gene dosage), and protein processing.<sup>8</sup> For many genes, small fluctuations in the amount of the gene product can turn gene expression on or off through feedback mechanisms. For other genes, even large fluctuations do not alter gene expression. The clinical manifestations of altered gene expression can vary greatly as well. Some disorders can arise from small fluctuations in gene product expression, whereas no clinical consequences may be apparent from large fluctuations in some gene products.

Determining where genes are located on the chromosome (mapping) and characterization of DNA requires generation of a sufficient quantity of DNA fragments. Portions of DNA containing a specific gene are removed from the chromosome by using enzymes that recognize specific base-pair sequences (restriction enzymes). The number of DNA fragments is then amplified (increased) and cloned (duplicated) in prokaryotic cells (e.g., bacteria), in eukaryotic cells (e.g., yeast), or by using the polymerase chain reaction (PCR). PCR is an efficient and highly sensitive method that allows the in vitro synthesis of large amounts of a desired DNA sequence. In addition, many other powerful molecular biology techniques have been developed to help researchers and clinicians identify the genetic link to human diseases. To learn more about molecular biology, the reader is referred to several recent review articles.<sup>9-10</sup>

**Figure 2.** Production of proteins from genes. The production of a protein product is a highly regulated, complex process that starts with the gene. At step 1, transcription of DNA into RNA starts upstream from the coding sequences and continues along the chromosome including both exon and intron sequences. At step 2, the primary RNA transcript is then modified to remove the areas transcribed from intron. At step 3, the resultant messenger RNA is then transported from the nucleus into the cytoplasm where, at step 4, it is translated into the amino acid sequence for the desired protein. Step 5 shows assembly of the completed protein. (From Thompson MW, McInnes RR, Willard HF. *Thompson genetics in medicine*. 5th ed. Philadelphia: Saunders; 1991. Adapted by permission.)



### Gene Delivery into Cells

Gene therapy requires introduction of foreign DNA sequences with stable integration, gene expression, and appropriate regulation in target tissues. The introduced gene can replace a missing gene or augment a defective one. For a gene to be expressed, it must first be delivered into the target cell. Once the gene is incorporated into the cell nucleus, the cell can then produce the new or missing gene product. Theoretically, genes can be transferred into many different cell types. Experimental techniques for delivering genes into hepatocytes, keratinocytes, fibroblasts, endothelial cells, epithelial cells, myocytes, and hematopoietic cells are available.<sup>11-17</sup> Most gene transfer experience has been with hematopoietic cells, since bone marrow or blood is easy to obtain and handle.<sup>13,18</sup> In particular, lymphocytes were used in the initial gene therapy experiments for adenosine deaminase (ADA) deficiency and cancer.<sup>19</sup> Unfortunately, while bone marrow stem cells are self-renewing, lymphocytes have a finite life cycle. Therefore, if lymphocytes are used as a cellular target for gene therapy, genes are expressed only as long as the lymphocyte lives. Thus, repeated infusions of gene-altered lymphocytes are required.

The currently available techniques for transferring genes into cells include both physical and viral meth-

ods.<sup>20,22</sup> Physical transfection methods include microinjection of DNA directly into cells, electrophoretic transfer across cell membranes (electroporation), coprecipitation with calcium phosphate, and fusion to liposomes or spheroplasts. Microinjection, although 10-100% efficient, is limited in clinical practice by the number of cells that can be injected. With microinjection, each individual cell would need to be treated separately and since  $10^6$  to  $10^9$  cells usually need to be treated, this method would not easily apply to many clinical situations, including bone marrow transplantation.<sup>20,21</sup> Other physical methods have limitations that make them less than optimal for current attempts at human gene therapy. Transfection and electroporation are inefficient; less than 1% of cells will have stable DNA integration into their chromosomes with this method. Fusion methods, using spheroplasts developed from bacteria after cell wall lysis or liposomes, involve attachment to the target cell membrane and then intracellular DNA delivery. DNA has been directly transferred to tumor cells via liposomes.<sup>21,24</sup> Although stable integration of DNA in the tumor cells may not be possible using liposomes, it may not be required since transient expression may be sufficient to exert a biological effect.

Viral vectors, primarily retroviruses, improve the efficiency of delivering genes into cells (Table 1).<sup>20,22,25</sup>

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A vector is any material containing DNA or RNA that is used to carry a gene into a cell. A viral vector provides a means of inserting genetic information into the genome of host cells. The Moloney murine leukemia virus is the most commonly used retrovirus (Figure 3). To provide the necessary safety factors, the retroviral vector is made replication-deficient (also referred to as replication-defective) by removing the encapsidation ( $\psi$ , *psi*) gene sequences necessary for viral replication. The vector can still infect the cell, integrate into the target cell, and deliver genetic information to the nucleus, but it cannot replicate. Viral proteins can then be made by the use of retrovirus "packaging" cell lines (Figure 4).<sup>26</sup> To accomplish this, the vectors are first modified to include the new gene(s) as well as the mechanism to control gene expression.

These genetically modified retroviruses have been used safely in humans for ex vivo gene therapy (Figure 5).<sup>2,19,27,28</sup> Scientists can transduce large numbers of cells by using this method.<sup>20,25</sup> Retroviral vectors provide an advantage over other vector systems because they can stably insert a foreign gene into the host genome. This modification would then be passed on as the cell divides. Retroviral vectors provide a highly efficient means of gene transfer (up to 90%) into replicating cells and precisely integrate transferred genes into cellular DNA.<sup>20,25</sup> Other methods of gene transfer are not as efficient as retroviral transduction and usually do not cause stable DNA integration into target cells, especially primary somatic (nongermlinal) cells.<sup>29</sup> However, retroviral vectors do not integrate in nondividing cells.<sup>30</sup>

Adenoviruses provide another viral vector for gene therapy.<sup>20,22,31</sup> Although not studied as extensively as retroviruses, they may play an important role in future gene therapy studies. After the genetic information controlling viral replication is removed from the adenovirus, it can be suitable for gene therapy in a manner

similar to the retrovirus. However, adenoviral DNA functions in an extrachromosomal manner, rather than by insertion into the genome. Because of this, the adenovirus should not cause malignant transformation (insertional mutagenesis or insertional oncogenesis). Unfortunately, the gene cannot be passed on to the progeny of the modified cell. Adenoviral vectors may cause transient high-level expression of genes in many cell types and could be useful in short-term gene therapy. The major advantages for using adenoviral vectors are that they are suitable for infecting tissues in situ, especially the lung, and they can be made at high titers ( $10^{11}$ – $10^{12}$  plaque-forming units/mL), thereby allowing for high-efficiency transduction. Cells in the lung proliferate slowly and many are terminally differentiated, making them less susceptible to retroviral vector transduction. One example of the current use of an adenoviral vector is to deliver a human cystic fibrosis transmembrane conductance regulator gene by intratracheal instillation to airway epithelial cells.<sup>32,33</sup>

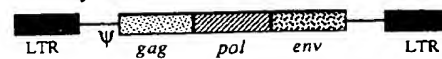
The herpes simplex virus (HSV) is another viral vector with a natural affinity (tropism) for nondividing tissue. This virus is neurotropic and may be suitable

**Figure 3.** Retroviral gene structure. The Moloney murine leukemia virus is an attractive starting point to produce retroviral vectors with substituted gene sequences of interest because the *gag*, *pol*, and *env* regions can be deleted and the virus can still enter a cell and integrate its genetic material without being able to reproduce. Dual-gene (or multiply-substituted) vectors can be produced by inserting the appropriate promoter sequence before each additional gene. The LNL6 NeoR retroviral vector is a highly modified version of previous NeoR vectors to enhance safety: a stop codon (TAG) replaces the start codon, and murine sarcoma virus sequences (hatched regions) replace some Moloney virus sequences. LTR = long-terminal repeat,  $\psi$  (*psi*) = encapsidation sequence, *gag* = viral structural-protein sequences, *pol* = viral enzymes (including reverse transcriptase) sequence, *env* = viral envelope-protein region, P = gene promoter, TAG = thymidine-adenine-guanine stop codon triplet, NeoR = gene that confers resistance to the neomycin analogue G418. (From Cornetta K. Safety aspects of gene therapy. *Br J Haematol.* 1992; 80:421-6. Adapted with permission of Blackwell Scientific Publications Ltd.)

Table 1.  
Viral Vectors and Target Tissues

Viral Vector	Target Tissues
Retrovirus	Hematopoietic cells Hematopoietic stem cells Fibroblasts Endothelial cells Myoblasts Smooth muscle cells Hepatocytes
Adenovirus	Airway epithelial cells Hepatocytes Hematopoietic cells (lymphoid, myeloid)
Adeno-associated virus	Hematopoietic cells Fibroblasts Epithelial cells
Herpesvirus	Lymphoid cells Nondividing cells (e.g., differentiated neurons and hepatocytes)
Parvovirus Simian virus 40	Central nervous system Smooth muscle cells

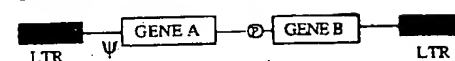
#### Moloney Murine Leukemia Virus



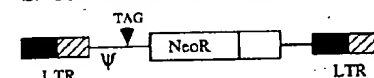
#### Single-Gene Retroviral Vector



#### Dual-Gene Retroviral Vector

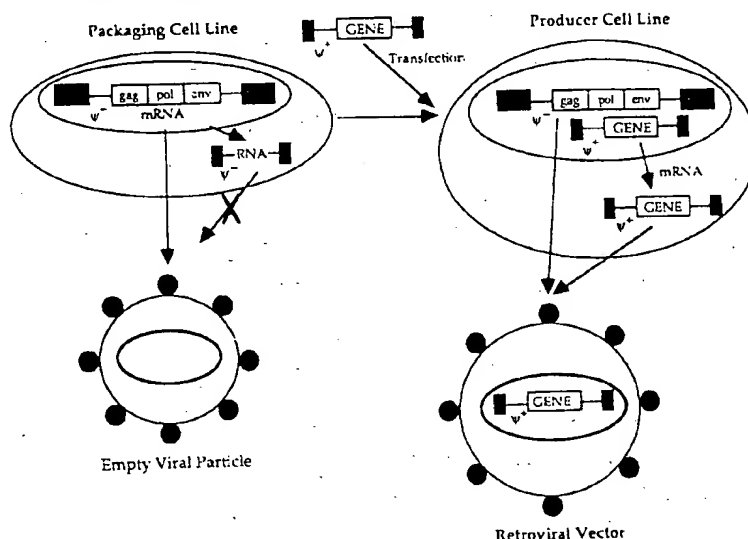


#### LNL6 NeoR Retroviral Vector

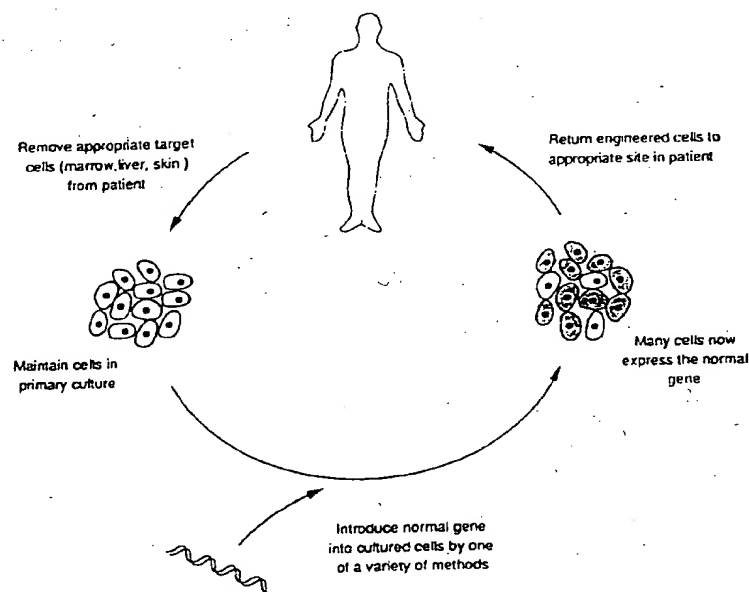




**Figure 4.** Gene insertion using retroviral vectors and packaging cell lines. A packaging cell line is used to produce replication-deficient retroviral vectors. Lacking the  $\psi$  ( $\psi$ ) viral encapsidation sequence, the packaging cell line cannot incorporate the viral genome into the virion and produces an empty viral particle. If this cell line is then transfected with a  $\psi$ -positive gene (cDNA sequence), the viral genome is preferentially packaged into the particles. (From Cornetta K. Safety aspects of gene therapy. *Br J Haematol*. 1992; 80:421-6. Adapted with permission of Blackwell Scientific Publications Ltd.)



**Figure 5.** Ex vivo gene therapy in humans. (From Valle D. Treatment of genetic disease: current status and prospects for the future. *Semin Perinatol* 1991; 15(Suppl 1):52-6. Adapted with permission.)



for diseases of the central nervous system.<sup>24</sup> A unique characteristic of HSV that may offer advantages for future gene therapy applications is that there is space available to accommodate larger and more complex gene arrangements than adenoviral and retroviral vectors.<sup>22</sup> HSV has not been used as a vector system in human studies yet.

The parvovirus, an adeno-associated virus (AAV) that is nonpathogenic and replication-defective, is being studied as a gene vector.<sup>22</sup> It has a broad range of possible hosts and can integrate into a specific site in its host's DNA, thus minimizing the risk of insertional mutagenesis. If necessary, introduced sequences may be removed by using a helper virus. The ability of the

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AAV to enter latency is attractive for AAV vector development.

### Potential Target Tissues

Although many types of tissue would theoretically be appropriate targets for correction of genetic defects, placing a normal gene into diseased tissues depends on several factors, including the proliferative state of the tissue, its accessibility to gene manipulation, the normal site of gene expression, the presence of cofactors, and the reversibility of tissue damage. Hematopoietic cells are favored since there are well-developed procedures for bone marrow transplantation (BMT), several hematopoietic cell types, wide distribution of hematopoietic cells, and a variety of severe diseases affecting hematopoietic cells.<sup>20,22</sup>

For many genetic therapies, the long-term, repopulating pluripotent hematopoietic stem cell is the ideal target for gene transfer.<sup>18,35,36</sup> Because of its extensive self-renewing capacity, a lasting genetic modification of the hematopoietic system can be accomplished by gene transfer.<sup>18,35</sup> Unfortunately, the hematopoietic stem cell is present in low concentrations, so many current gene therapy efforts target more differentiated hematopoietic cells, such as lymphocytes.<sup>15</sup> However, recent advances have made gene transfer into hematopoietic stem cells possible, and current research efforts are under way to make this approach more feasible.<sup>18,35,36</sup>

The liver is also an attractive target to correct the many genetic disorders associated with inborn errors of metabolism.<sup>21,37,38</sup> The liver has a blood supply that can be isolated for gene delivery and then used to distribute the gene product. While the hepatocyte does not proliferate to the extent that other target tissues for gene therapy (e.g., lymphocytes) do, it undergoes sufficient divisions to allow a 3–30% transduction rate.<sup>21,37,38</sup> Furthermore, hepatocytes can be removed by partial liver resection, grown in tissue culture, and then returned via the hepatic artery. While in culture, they can be transduced with recombinant retroviral vectors where, as has been demonstrated, a functional defect can be repaired.<sup>35</sup> Moreover, there are a number of diseases that result from hepatic deficiency states. It may be possible to cure a genetic disorder by supplementing the liver with a gene that is deficient in specific disease states. There is interest in the potential application of gene therapy in antitrypsin deficiency, a common hepatic deficiency state that leads to early-onset lung disease.<sup>2,33</sup>

Many metabolic, exocrine, autocrine, and nutritional disorders may be treated in the future by gene therapy targeting the intestines. Preliminary evidence suggests that intestinal tissue can be transduced by exposure to adenoviral vectors. Other potential targets for gene therapy in the gastrointestinal tract include the bile duct, pancreatic cells, and other secretory cells.<sup>25</sup>

Investigators have transferred the gene encoding tissue plasminogen activator (TPA) into endothelial cells

via retroviral vectors: The genetically modified endothelial cells will then express TPA. Although a systemic anticoagulant effect would not be expected from this production, local concentrations of TPA could produce a thrombolytic environment and reduce thrombosis.<sup>39</sup>

Most investigators agree that protocols modifying somatic tissues, as described above, are an ethical therapeutic option. However, controversy still exists as to the ethics of using germ cells (e.g., sperm or eggs) for gene therapy. Future generations could be damaged if the germ line is manipulated. Much is still not known about this type of therapy. Long-term adverse effects may occur if genetic information in patients' germinal cells is altered. Therefore, before gene therapy in human germ cells is considered, more experience is necessary in animal models.<sup>40</sup>

Although preliminary research indicates promise in many of the previously described tissues, formidable obstacles need to be overcome before we see routine use of gene therapy in human disease. In the future, it may be possible to use gene therapy in a variety of cell types as a means of creating customized, localized, drug delivery systems in patients by producing drugs targeted at specific disease processes.

### Disease Candidates for Gene Therapy

As scientists make progress in gene transfer techniques and uncover more details of the human genome, the list of potential applications of gene therapy will grow (Table 2). Ethical and scientific considerations are important when choosing initial candidates for gene therapy. At first, clinicians considered severe genetic diseases with a predictable phenotype and

Table 2.  
Potential Disease Candidates for Gene Therapy

Genetic diseases or disorders with associated genetic component
SCID <sup>a</sup> with adenosine deaminase deficiency
Familial hypercholesterolemia
Hemophilia
Cystic fibrosis
Antitrypsin deficiency
Chronic granulomatous disease
Gaucher's disease type I
Mucopolysaccharidosis
Emphysema
Phenylketonuria
Ammoniaemia
Muscular dystrophy
Thalassemia
Sickle cell anemia
Argininosuccinic aciduria
Citrullinemia
Purine-nucleoside phosphorylase deficiency
Cancer
Nongenetic, nonmalignant diseases
Acquired immunodeficiency syndrome
Cardiovascular disease

<sup>a</sup>SCID = severe combined immunodeficiency.

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limitations in current therapy that justified experimental approaches. The basic categories of disorders in which gene therapy will be used are (1) diseases caused by a missing or defective gene, (2) cancer, and (3) nongenetic, nonmalignant diseases such as AIDS.

To correct genetic disorders using the current gene transfer systems, several biological criteria should be met: (1) the disease is genetically recessive, (2) the defective gene has been identified and cloned, (3) precise regulation of gene product is not required, and (4) target cells can be manipulated after removal from the body and safely returned to the patient.<sup>1,2</sup> Furthermore, cells containing the corrected gene(s) should have a selective survival advantage over uncorrected cells. Without the survival advantage, remaining untreated cells would dilute the beneficial effects of the genetically corrected cells. Recent advances in recombinant DNA technology, which make isolation of many genes possible, and new insights into gene regulation mechanisms make meeting these biological requirements feasible.

At present, the most suitable genetic disorders meeting these criteria are those affecting the hematopoietic system. This is because a suitable method for reintroducing genetically altered cells already exists through BMT. In general, BMT is successful in diseases in which (1) the defect causes a complete or partial lack of functioning cells in a particular cell line, (2) there is defective enzyme synthesis, or (3) there is a defective transport molecule. These situations could be corrected by replacing the defective gene with its cloned functional equivalent. This approach is successful in treating hematopoietic disorders like severe combined immunodeficiency due to ADA deficiency.<sup>41,42</sup> Gene therapy may prove more difficult in disorders that involve complex gene regulation (e.g., diabetes mellitus) or where the disease is manifested in relatively inaccessible body tissues, such as the bone matrix or the central nervous system (CNS). With current technology, genes cannot be supplied through hematopoietic pathways to many other tissues, such as muscle, visceral organs, or neurons, and alternative gene delivery pathways are necessary. CNS disease may prove to be particularly difficult to treat. In the CNS, only a relatively small number of diseases may be correctable with currently anticipated technologies. When a gene deficit results in a toxic product, early developmental failure caused by accumulation of toxic metabolites in the CNS may be irreversible. Damage to neurons may also result from physical damage induced by gene insertion (i.e., an immune response to a herpes vector). For these reasons, gene therapy of many CNS diseases may need to take place in the fetus or newborn before irreversible damage occurs and where the blood-brain barrier may be more permeable to treated cells.

Gene therapy is usually thought of as a technique for inserting a functioning gene into cells of a patient to correct an inborn genetic defect.<sup>1</sup> Gene therapy for malignant disease, for the most part, consists of inserting a gene into a cell to provide a new function for the

cell. Newer approaches are targeting the genetic basis for many malignancies by introducing missing tumor suppressor genes or inactivating oncogenes.<sup>43</sup> Current approaches to gene therapy for cancer involve gene transfer into immune cells to modify and enhance immune cell functions, modifying tumor cells to stimulate the immune response, inserting genes to make cancer cells sensitive to certain drugs (e.g., ganciclovir sodium), and using gene transfer for marking or enhancing transplanted bone marrow for treatment of lymphoid tumors.<sup>44</sup> Also, genetic marking of autologous bone marrow cells used for transplantation provides a way to determine if the transplanted (reinfused) cells are the cause of posttransplant relapse. Consequently, new ways of reducing residual disease before removal of bone marrow or more rigorous marrow purging could be evaluated.

The gene encoding bacterial neomycin phosphotransferase (usually referred to as the NeoR gene) has been successfully used in gene-marking studies.<sup>45</sup> Cells that produce neomycin phosphotransferase are resistant to the neomycin analogue G418 and can be distinguished from cells that do not express this enzyme. Insertion of the gene NeoR into tumor-infiltrating lymphocytes (TIL) demonstrated that the gene could be inserted and expressed in human TIL and that the marked cells could be detected in blood and tumor samples.<sup>45</sup> Studies done in a limited number of patients have now shown that these gene-modified cells can be detected for up to six months in blood and two months in tumor after cell infusion.<sup>45</sup> The NeoR gene is also being inserted into bone marrow cells used in BMT gene-marking studies.

Gene therapy has been proposed as a treatment for AIDS. Better understanding of modes of transmission and molecular mechanisms of human immunodeficiency virus (HIV) replication has yielded creative approaches to therapy. Gene transfer protocols have been designed to protect modified cells by blocking viral infection or by interfering with HIV gene expression. Investigators are searching for ways to protect T lymphocytes, the primary target of HIV, from infection. Protection of even a small proportion of T cells may salvage immune function in affected individuals and allow normal immune mechanisms to prevent further viral spread.<sup>46</sup>

Ultimately, investigators will have to look at practical issues to determine which current gene therapy strategies will address realistic clinical goals. Further research with viral vectors is needed to achieve both efficient gene transfer into target cell populations and stable expression of foreign gene products in clinically useful concentrations.

### Safety of Gene Manipulation

**Importance of Assessment.** Safety assessment is important with any new technology, but it is especially important for gene therapy studies, where, in some cases, retroviruses are intentionally introduced. All

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clinical studies involving gene transfer in human subjects are assessed by local institutional and national review committees. The local institutional review board evaluates the scientific and ethical merit of the study, and the institutional biosafety committee reviews the recombinant DNA aspects. In addition, all gene therapy protocols supported by U.S. government funds were initially reviewed by both the Human Gene Therapy Subcommittee (HGTs) of the Recombinant DNA Advisory Committee (RAC) and then separately by the RAC itself. Once gene therapy trials began and experience was gained, the need for this dual review by the HGTs and the RAC was questioned and the HGTs was subsequently dissolved; now, only the RAC review is required. The RAC acts in an advisory capacity to the director of the National Institutes of Health (NIH; Bethesda, MD), who ultimately grants final approval. For example, the first gene transfer study underwent 15 separate reviews before final approval was granted in January 1989. Most recently, the RAC has adopted a procedure for the compassionate use of gene therapy.<sup>47-49</sup>

During its existence, the HGTs produced a "Points To Consider" document to guide researchers in seeking approval for gene therapy studies.<sup>50</sup> Several points contained in this document deal directly with safety issues. These concern the need for special precautions to prevent the spread of the recombinant DNA, to reduce any potential hazards to persons other than the person being treated, and to explain and provide laboratory evidence regarding the potential harmful effects of the gene transfer (i.e., development of neoplasms, harmful mutations, regeneration of infectious particles, or an immune response). In considering retroviral-mediated gene transfer, the first safety point deals with the construction of a safe viral vector system. Often, this point forms the basis for most of the questions posed during the RAC review to investigators who want to perform gene transfer studies in humans. The investigators must demonstrate that they have considered all potential consequences of their study. The investigators must consider the potential for gene insertion into reproductive cells (e.g., sperm or eggs) and address the concern for retroviral infection of people other than the patient, including health care workers and family members.<sup>51</sup> FDA has produced a similar "Points To Consider" document that establishes the guidelines by which the Center for Biologics Evaluation and Research evaluates gene therapy studies.<sup>52,53</sup>

In the safety assessment of any gene therapy protocol using retroviral vectors, concern about potential harm to the patient, family, health care providers, and the general public needs to be addressed. Potential patient harm can include an increased risk of cancer and exposure to replication-competent retroviruses through recombination. Risks to those who come in close contact with the patient, such as family or health care providers, can include infection from infectious viral particles. The potential harm to the general popu-

lation includes the possible production of a new and infectious virus and the possible evolutionary effects of inadvertent retroviral infection of the germ line.<sup>54</sup>

**Retroviral-Mediated Gene Transfer.** Since most concern focuses on the most commonly used gene transfer system—retroviral vectors—it is important to understand the potential safety issues surrounding retroviral-mediated gene transfer.<sup>55-57</sup> These include (1) contamination with replication-competent virus, (2) contamination with pathogens or toxins, (3) potential problems associated with gene insertion into the host genome, and (4) specific problems related to the method by which the gene-altered cells are administered (e.g., hepatectomy) or the intended gene product (e.g., interleukin-2).

The most critical issue in the development of retroviral-mediated gene transfer was the assurance that replication-competent viruses would not be produced. The retroviral vectors are constructed lacking the *gag*, *pol*, and *env* gene sequences necessary for replication. Virion packaging is accomplished by using "packaging cell lines" rather than potentially infectious helper viruses.<sup>58</sup> Initially, these packaging cell lines were produced with a *psi* gene sequence deletion. It was soon learned that these cell lines can develop replication-competent virus through recombination of the *psi* sequence from the vector with the *psi*-negative helper virus. Newer packaging cell lines have been developed that lack both the *psi* gene sequence and parts of the long-terminal repeats at the 5' and 3' termini, thus necessitating two recombination events to produce a replication-competent virus. In addition, vectors have been produced that replace the codon for starting *gag* gene transcription with a codon for stopping transcription. Researchers have also used vectors with substituted sequences from viruses that are not structurally homologous to the helper virus to minimize the frequency of recombination. Further modifications of the retroviral vector and the packaging cell line have produced systems that require three separate recombination events, thereby greatly reducing the chance of producing a replication-competent virus.<sup>55-57</sup> More importantly though, no problems associated with replication-competent virus have been reported in the more than 110 patients treated in gene therapy trials to date.

Original observations suggested that murine retroviruses capable of replicating in the cells of other species in addition to the original host (amphotropic retroviruses) proliferate poorly in primates, suggesting that if replication-competent helper virus infection did occur, the clinical consequences would be minimal or nonexistent. Recently, however, Donahue et al.<sup>58</sup> reported the rapid occurrence of T-cell lymphoma in three profoundly immunosuppressed monkeys intentionally exposed to replication-competent helper virus used in the supernatant for a gene transfer experiment. The monkeys were given fluorouracil and their bone marrow was harvested, then they were irradiated and the bone marrow transplanted. PCR analysis clearly

showed that the malignant cells contained sequences homologous with the helper virus and did not contain sequences from the retroviral vector. This observation stresses the importance of using supernatant that is free from replication-competent helper virus for human gene therapy studies. Given the unique circumstances surrounding this report, the current packaging systems and assay methods still appear to be safe for human use, although improvements in both would be beneficial.<sup>59</sup>

Although precautions are taken to use the safest retroviral vectors and packaging cell lines available, there is still the chance that replication-competent virus can be transferred to the human genome. Researchers have developed sensitive tests to detect virus contamination. These tests include viral amplification in receptive (permissive) cells, which is 10–1000 times more sensitive than currently used S'/L<sup>-</sup> biologic or reverse-transcriptase assays, and the most sensitive test, PCR, which can detect one infected helper virus out of 100,000 transduced cells.<sup>54,56</sup> Before introduction of the transduced cells into the patient, standard FDA tests for contamination are performed on the producer cell line, the viral supernatant, and the transduced cells themselves. This minimizes the potential for infecting the patient with other pathogens or toxins.<sup>52,53</sup>

There are several additional concerns relating to foreign gene integration into the host genome. Retroviruses and retroviral vectors insert randomly into the chromosomes with a possible preference for transcriptionally active regions.<sup>55,56</sup> The potential problems that may result from this insertion are related to the insertion position and normal function of the original cellular gene. If the retrovirus integrates into a control or coding sequence, or possibly even into untranslated, untranscribed, or intervening sequences of an essential gene, it may cause inactivation or disruption of that gene's normal regulation. These alterations may result in no effect, individual cell death, or malignant transformation.<sup>56,57</sup>

The most important concern is that retrovirus insertion may activate a proto-oncogene or inactivate a tumor suppressor gene, resulting in malignant transformation. The probability of malignant transformation is related to the number of cells infected, the number of integrations per cell, the presence of proto-oncogenes, the role of suppressor genes, the number of cellular changes required to cause malignancy, the virus's natural tropism for host cells, environmental factors, and the efficiency by which these insertions operate. It is estimated that as many as 10 separate factors may be involved in the development of certain malignancies.<sup>56,60</sup> Thus, retroviral-mediated gene transfer, which in some cases is designed to insert only one vector per cell, will have a very low probability of causing all of the cellular changes needed to induce malignant transformation.<sup>56,57,60</sup>

It is known, however, that certain human retroviruses can produce malignancies such as T-cell lymphoma

or AIDS. The absolute risk of cancer development with murine retroviruses or murine-derived retroviral gene transfer to humans is very low, but it cannot be accurately determined until more experience is gathered by using these techniques. Also, there is a risk for secondary malignancy development when radiation therapy or many chemotherapeutic agents (e.g., alkylating agents) are used for treating cancer. Whether the risk for secondary malignancy is any higher (if it exists at all) with retroviral-mediated gene transfer is yet to be determined. No evidence of malignant transformation has been seen in any of the humans treated since gene transfer trials began in 1989.<sup>40</sup>

**Toxicities of Concurrent Therapies.** The potential toxicities associated with other biological agents given to support the growth of the gene-altered cells should also be considered. For instance, if the transduced cells need to be given in the presence of aldesleukin (recombinant human interleukin-2), then consideration must be given to the potential combined toxicities related to aldesleukin and the gene product. Likewise, if the gene-altered cells are to be returned to the patient through BMT, then the adverse effects associated with the high-dose antineoplastic preparative regimen given before BMT should also be addressed. Consideration must also be given to the potential toxicities of the intended gene product. For example, if the gene for tumor necrosis factor (TNF) is being introduced into a lymphocyte, consideration must be given to potential systemic TNF toxicities, even if the therapy is intended to be localized.<sup>51,58</sup>

In the first government-approved trial in humans, the NeoR gene, which has no intrinsic therapeutic properties, was used as a marker to identify transduced cells to demonstrate the safety and feasibility of ex vivo retroviral-mediated gene transfer. In this study, 10 patients with malignant melanoma were treated with NeoR-transduced TIL. As in prior TIL studies, aldesleukin was given to patients concurrently with the TIL to support their in vivo growth.<sup>34,45</sup> Therapy-related adverse effects were no different from those normally seen with TIL plus aldesleukin therapy. Rigorous testing showed that the viral supernatant used for gene transduction and TIL was sterile and contained no helper virus. PCR analysis and reverse transcriptase assays demonstrated no amphotropic helper virus in the infused TIL. Results of Western blot analysis for viral gag protein and S'/L<sup>-</sup> assay for virus, performed on the patients' serum multiple times throughout the study up to 180 days after cell infusion, were negative. Thus, it was demonstrated for the first time that ex vivo retroviral-mediated gene transfer into T lymphocytes, under the proper conditions, did not produce evidence of disease after infusion in humans. Similar safety assessments with adenoviral vectors are currently in progress.

**Safety Record.** The accumulated experience with retroviral-mediated gene transfer from the equivalent of 106 monkey-years and 23 patient-years has demonstrated no adverse effects, no pathologic effects, and

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no malignancy development related to gene transfer.<sup>40</sup> However, as with any new experimental therapy, the potential risks associated with human gene therapy, including the risk of cancer development, must be judged against the potential toxicities associated with alternative treatments and disease severity.

### Clinical Applications of Gene Transfer and Gene Therapy

The possible applications of human gene therapy to treat human diseases are virtually limitless.<sup>1,2,20,61,62</sup> Initial studies were directed at correcting inherited disorders and were subsequently expanded to better understand and possibly treat malignant diseases. Clinical studies for nongenetic, nonmalignant diseases have recently begun. In some trials (gene-marking studies), cells are genetically marked so that they can be tracked throughout the body to enhance understanding of disease biology (Table 3). In other studies, a gene is transferred as a means of treating a human disease; these studies are referred to as gene therapy studies (Table 4). Gene-marking and gene therapy studies are beginning to span the globe, with ongoing trials in Italy, France, Netherlands, China, England, and Germany.

#### Genetic Disorders or Disorders with Associated

**Genetic Component.** For only a few of more than 4000 known human genetic disorders have the responsible genes been cloned to allow possible correction with gene therapy.<sup>27,63-66</sup> Clinical-gene therapy trials have already been initiated in severe combined immunodeficiency syndrome (SCID) associated with ADA deficiency, hemophilia B, familial hypercholesterolemia associated with a defect in the low-density-lipoprotein (LDL) receptor gene, and cystic fibrosis.

**ADA-Deficient SCID.** Researchers considered ADA-deficient SCID to most closely fit the criteria for an ideal candidate for genetic therapy.<sup>19,41,42</sup> The first human gene therapy attempt took place on September 14, 1990, in the pediatric intensive care unit at the Warren G. Magnuson Clinical Center of NIH. A four-year-old girl with ADA-deficient SCID and persistent immunodeficiency despite pegademase bovine therapy for more than two years was given a transfusion of her own peripheral-blood T lymphocytes that had been transduced *ex vivo* using retroviral-mediated gene transfer with the gene encoding normal human ADA.<sup>19,40</sup> It was hoped that restoration of ADA enzyme activity would restore cellular immunity and allow this child to return to a more normal life.

She received 11 infusions over the next two years, resulting in her regaining and maintaining some normal immune functions. Her intracellular ADA concen-

Table 3.  
Gene-Marking Studies with the Neomycin Resistance (NeoR) Gene

Month First Patient Started	Institution <sup>a</sup>	Condition	Cells Marked <sup>b</sup>
5/89	NIH	Malignant melanoma	TIL
9/91	St. Jude	Pediatric acute myelogenous leukemia	Bone marrow
12/91	Centre Leon Berard	Malignant melanoma	TIL
1/92	St. Jude	Neuroblastoma	Bone marrow
3/92	Pittsburgh	Malignant melanoma	TIL
5/92	Indiana	Adult acute leukemias	Bone marrow and autologous peripheral blood
7/92	M. D. Anderson	Chronic myelogenous leukemia	Bone marrow and PBSC <sup>c</sup>
9/92	NIH	Multiple myeloma or chronic myelogenous leukemia	Bone marrow and PBSC <sup>c</sup>
12/92	NIH	Breast cancer	Bone marrow and PBSC <sup>c</sup>
2/93	UCLA	Malignant melanoma or renal cell cancer	PBL and TIL (CD4 <sup>+</sup> and CD8 <sup>+</sup> subsets)
Pending	Baylor	Liver failure	Hepatocytes
Pending	NIH	HIV <sup>d</sup> infection	Syngeneic CD4 <sup>+</sup> and CD8 <sup>+</sup> T lymphocytes <sup>e</sup>
Pending	M. D. Anderson	Chronic lymphocytic leukemia	Bone marrow and PBSC <sup>c</sup>
Pending	Fred Hutchinson	Nonmyeloid malignancies	IL-3 or G-CSF-stimulated PBSC
Pending	St. Jude	Neuroblastoma	Purged and unpurged bone marrow
Pending	St. Jude	Pediatric acute myelogenous leukemia	Bone marrow purged by two different methods <sup>c</sup>
Pending	St. Jude	Bone marrow transplant	EBV-specific cytotoxic T lymphocytes

<sup>a</sup>Full names and locations are National Institutes of Health (NIH), Bethesda, MD; St. Jude Children's Research Hospital, Memphis, TN; Centre Leon Berard, Lyon, France; University of Pittsburgh, PA; Indiana University, Indianapolis; The University of Texas M. D. Anderson Cancer Center, Houston; Baylor University, Waco, TX; University of California-Los Angeles (UCLA); Fred Hutchinson Cancer Center, Seattle, WA.

<sup>b</sup>TIL = tumor-infiltrating lymphocytes; PBSC = peripheral-blood stem cells; PBL = peripheral-blood leukocytes; IL-3 = interleukin-3; G-CSF = granulocyte colony-stimulating factor; EBV = Epstein-Barr virus.

<sup>c</sup>Marked with G1Na and LNL6 NeoR vectors.

<sup>d</sup>HIV = human immunodeficiency virus.

Table 4.  
Gene Therapy Studies\*

Month First Patient Started	Institution <sup>b</sup>	Disease	Transferred Genes	Target or Delivery Cells
9/90	NIH	ADA-deficient SCID	ADA	PBTC or PBSC
1/91	NIH	Malignant melanoma	TNF	TIL
10/91	NIH	Advanced cancer	TNF	Tumor
12/91	Fudan-Changhai	Hemophilia B	Factor IX	Autologous skin fibroblasts
2/92	Leiden	ADA-deficient SCID	ADA	Bone marrow and PBL
3/92	San Raffaele	ADA-deficient SCID	ADA	PBTC and progenitor-enriched bone marrow
3/92	NIH	Advanced cancer	IL-2	Tumor
6/92	Michigan	Malignant melanoma	HLA-B7	Melanoma in vivo via liposomes
6/92	Michigan	Familial hypercholesterolemia	LDL receptor	Hepatocytes
12/92	St. Jude	Relapsed or refractory neuroblastoma	IL-2	Tumor
12/92	NIH	Primary or metastatic brain tumor	HSV-TK	Tumor in vivo
2/93	MSKCC	HLA-A2-positive malignant melanoma or renal cell cancer	IL-2	Allogeneic tumor
2/93	Washington	AIDS-related lymphoma	HSV-TK and HPH	CD8 <sup>+</sup> HIV-specific cytotoxic T lymphocytes
3/93	Netherlands	ADA-deficient SCID	ADA	Bone marrow
4/93	NIH	Cystic fibrosis	CFTR <sup>c</sup>	Respiratory epithelial cells via direct inhalation
Pending	Rochester	Ovarian cancer	HSV-TK	Ovarian cancer
Pending	Viagene	AIDS	HIV env	Fibroblasts
Pending	M. D. Anderson	Lung cancer	Antisense K-ras or p53	Tumor
Pending	Pittsburgh	Advanced cancer	IL-4	Autologous fibroblasts and Autologous tumor
Pending	Iowa	Cystic fibrosis	CFTR <sup>c</sup>	Nasal epithelial cells via direct inhalation
Pending	Michigan	Cystic fibrosis	CFTR <sup>c</sup>	Lung segment
Pending	Iowa	Primary or metastatic brain tumor	HSV-TK	Tumor in vivo
Pending	Cincinnati	Cystic fibrosis	CFTR <sup>c</sup>	Respiratory epithelial cells
Pending	Johns Hopkins	Renal cell cancer	GM-CSF	Tumor
Pending	North Carolina	Cystic fibrosis	CFTR <sup>c</sup>	Nasal epithelial cells

\*ADA = adenosine deaminase deficiency; SCID = severe combined immunodeficiency; PBTC = peripheral-blood T cells; PBSC = peripheral-blood stem cells; TNF = tumor necrosis factor; TIL = tumor-infiltrating lymphocytes; PBL = peripheral-blood lymphocytes; IL-2 = interleukin-2; HLA = human lymphocyte antigen; LDL = low-density lipoprotein; HSV-TK = herpes simplex virus thymidine kinase; HPH = hygromycin phosphotransferase; HIV = human immunodeficiency virus; IL-4 = interleukin-4; CFTR = cystic fibrosis transmembrane conductance regulator; GM-CSF = granulocyte-macrophage colony-stimulating factor.

<sup>b</sup>Full names and locations are: National Institutes of Health (NIH), Bethesda, MD; Fudan University and Changhai Hospital (Fudan-Changhai), Shanghai, China; University Hospital, Leiden, Netherlands; San Raffaele Scientific Institute, Milan, Italy; University of Michigan, Ann Arbor; St. Jude Children's Research Hospital, Memphis, TN; University of Rochester, NY; Viagene (a for-profit research company), San Diego, CA; The University of Texas M. D. Anderson Cancer Center, Houston; Memorial Sloan-Kettering Cancer Center, New York, NY; University of Washington, Seattle; University of Pittsburgh, PA; University of Iowa, Iowa City; University of Cincinnati, OH; The Johns Hopkins University, Baltimore, MD; University of North Carolina, Chapel Hill.

<sup>c</sup>Disabled adenovirus construct.

tration rose from undetectable to 20–30% of the normal value. Her T lymphocyte count rose to normal value. Some other indicators of immune status also improved, including antibody response to blood group antigens (isohemagglutinin titers), response to some skin test antigens, and in vitro T lymphocyte responses to influenza and allogeneic cells. She is now doing well, is able to play outdoors and swim in the community pool, suffers fewer infections, and has entered public school. A second patient started treatment in January 1991 and is showing similar laboratory and clinical improvement.<sup>67</sup>

Since T lymphocytes are fully differentiated and therefore have a limited life span, it was thought that the beneficial effects of the transduced cells (if any) would be short-lived and that there would be gaps in the patient's immune repertoire. In the first patient treated, the infusions of transduced T lymphocytes were not given during a six-month period to deter-

mine the half-life of the infused cells. The number of peripheral-blood T lymphocytes gradually fell toward baseline over the six months, suggesting that the half-life was at least three months. Intracellular ADA concentrations did not decline during this period. These findings suggested that there is a selective survival advantage for the genetically corrected cells in vivo.

As a possible method of conferring lifelong gene correction, the NIH study has since been amended to transduce monoclonal-antibody-separated CD34<sup>+</sup> peripheral-blood stem cells harvested under the influence of filgrastim.<sup>68</sup> The transduced peripheral-blood stem cells will be marked with a different ADA vector, enabling the researchers to determine which cell type, peripheral-blood stem cell or peripheral-blood T lymphocyte, is providing the greatest benefit. A trial using this technique has already begun in Italy, with the first patient enrolled in March 1992.<sup>40,69</sup> It is hoped that stem cell transduction will produce lifelong correction

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of the enzyme defect and possibly become a one-shot cure for ADA deficiency and other genetic disorders. Other investigators are pursuing gene transfer into hematopoietic stem cells as well.<sup>18,35,36</sup>

**Hemophilia, Thalassemia, and Sickle Cell Anemia.** While the work with ADA deficiency has provided invaluable insight into the possibilities for gene therapy, ADA deficiency is exceedingly rare compared with other genetic disorders. A group of much more common genetic blood disorders, such as hemophilia, thalassemia, and sickle cell anemia, have been targeted for gene therapy.<sup>70-73</sup> A gene therapy trial for hemophilia B began in December 1991 in Shanghai, China.

Gene therapy approaches to the hemophilias are different from the approaches to ADA deficiency.<sup>70-73</sup> Hemophilias A and B are caused by a variety of mutations occurring in the genes encoding clotting factor VIII or IX, respectively, resulting in clotting factor deficiency. Ideally, the mutant gene would be repaired by splicing it with normal DNA. Although preliminary work has shown that this is possible in vitro, it is highly unlikely that this process will be available for in vivo use given the diversity of mutations encountered. As an alternative, researchers are investigating gene insertion techniques that allow cells to produce the missing factor(s). Although the liver might be the ideal target for correcting these hemophilias, much of the work thus far has been done by using transduced fibroblasts.

Preliminary evidence suggests that adequate factor IX production can be achieved in vivo by using these techniques; however, persistent gene expression is a problem that still needs to be solved. Studies initially done in mice have demonstrated that an immune response can be mounted against the newly produced human protein, indicating that gene replacement efforts may be more beneficial in patients who produce insufficient amounts of protein product, rather than no protein at all.<sup>61</sup> Clinical studies using gene therapy to correct the other genetic blood disorders have not started yet; these diseases present difficult obstacles to overcome because the missing or abnormal protein product comes from a series of complex, tightly regulated, gene-containing clusters and the optimal gene delivery system has not yet been determined.<sup>71,72</sup>

**Familial Hypercholesterolemia.** A gene therapy trial for the treatment of familial hypercholesterolemia, which affects young children, has recently begun. Familial hypercholesterolemia results from a defect in the LDL-receptor gene. Deficient LDL-receptor production results in a lack of LDL catabolism, causing high plasma concentrations of LDL and total cholesterol. This results in premature development of coronary artery disease. In this study, hepatocytes are removed from the patient and transduced with a retroviral vector containing a human LDL-receptor gene. Studies have demonstrated that this technique is feasible, with a transduction efficiency rate approaching 30%.<sup>21,37,74</sup> The transformed hepatocytes are then able to express sufficient quantities of recombinant LDL-receptor pro-

tein on the cell surface. It is hoped that this will serve as an adjunct therapy.<sup>37,38,74</sup> According to a preliminary report on the first patient treated, the transferred gene was functioning in the patient's liver, her plasma cholesterol concentration has fluctuated from 20% to 40% below her baseline values, she has experienced no untoward effects as a result of the therapy, and she was recently started on cholesterol-lowering agents (now that she has LDL receptors).<sup>75</sup>

**Cystic Fibrosis and Antitrypsin Deficiency.** Cystic fibrosis and antitrypsin deficiency are the two most common genetic pulmonary disorders in the United States.<sup>33,76</sup> Gene therapy may offer a promising approach for long-term treatment for both diseases.<sup>32,33,76</sup> Five cystic fibrosis gene therapy trials have recently been approved.

Cystic fibrosis is a disorder caused by a defect in the gene for the cystic fibrosis transmembrane conductance regulator (CFTR) protein, resulting in altered electrolyte transport in the epithelial cells of the pancreas, gastrointestinal tract, and tracheobronchial tree.<sup>33,76</sup> Alpha-1-antitrypsin is a neutrophil elastase inhibitor, and deficiency results from inadequate  $\alpha$ -1-antitrypsin production by the liver.<sup>33</sup> Neutrophil elastase is a protease that can destroy the connective tissue backbone of alveolar walls; thus, antitrypsin deficiency can result in progressive lung tissue destruction and emphysema. Infantile hepatic cirrhosis can also occur with antitrypsin deficiency.<sup>2,33</sup> A possible gene therapy approach to correct these disorders would be to deliver the CFTR or  $\alpha$ -1-antitrypsin gene directly into the epithelial cells lining the airways.<sup>32,33</sup> Alternatively, the  $\alpha$ -1-antitrypsin gene can be directed toward hepatocytes in a more classic gene replacement attempt.<sup>2</sup>

However, unlike other tissues targeted for gene therapy, lung tissue proliferates very slowly, making it less than suitable for using retroviral vectors. Investigators have developed a technique to directly administer the CFTR or  $\alpha$ -1-antitrypsin gene to airway epithelial cells by using an adenoviral vector.<sup>31,33</sup> As mentioned previously, an adenovirus-based vector was chosen because adenoviruses can efficiently infect nondividing lung epithelial cells. When adenovirus-based vectors carrying the normal CFTR gene were tested in cells from patients with cystic fibrosis, the infected cells demonstrated restoration of functional chloride channels.<sup>33</sup> A similar positive finding was demonstrated with vectors carrying the  $\alpha$ -1-antitrypsin gene. However, it should be noted that these localized strategies would correct only the pulmonary complications for these disorders and would not affect the hepatic complications associated with antitrypsin deficiency or gastrointestinal complications associated with cystic fibrosis.

Although this technique may pave the way for more direct in vivo gene transfer studies, whether it will work in human lung is yet to be demonstrated. Problems may arise in patients who have been previously exposed to adenovirus infection. If immunity devel-



ops, gene transfer efficiency may be compromised. If repeated inoculations are necessary to provide adequate viral episomes, mild immunosuppression may be necessary, which would be less than ideal in a patient already prone to infections.

**Hepatic Disease.** A soon-to-begin study will use retroviral-mediated gene transfer to mark hepatocytes as part of a hepatocellular transplant protocol.<sup>77</sup> It is thought that engrafting as little as 5% of normal hepatocytes into a diseased liver may restore enough function to provide time to find a suitable liver donor. Marking the donated hepatocytes with a retroviral vector, such as the NeoR gene, will allow researchers to track the progress of the hepatocytes and possibly optimize surgical techniques.

**Other Diseases.** Gene therapy is being explored for other diseases. It is closest to clinical trials for certain forms of chronic granulomatous disease (CGD) and Gaucher's disease, a hereditary lack of the enzyme used by lysosomes to break down glucocerebroside.<sup>66,78,79</sup> For patients with CGD, researchers are working to insert the gene responsible for superoxide production into bone marrow or peripheral-blood stem cells.<sup>79</sup> Two approaches are being explored for Gaucher's disease.<sup>66,78</sup> One investigator is working on transducing bone marrow stem cells to produce the deficient enzyme. Another is working on maintaining lowered plasma lipid concentrations by using bioengineered fibroblasts or endothelial cells.<sup>66,78</sup>

Much research is under way to examine the potential applications of gene therapy in treating many other genetic disorders. With the expanding possibility of transducing target tissues in vivo, increasing the efficiency of gene transfer, and specifically directing in vivo delivery of the genes, a dramatic increase in the application of gene therapy to correct genetic disorders should occur within the next 10 years.

**Cancer.** The discovery and application of biological therapy to treat human cancers has provided insight into the many possible approaches for using gene therapy to diagnose and treat cancer and to monitor cancer therapy.<sup>43,44,50,54</sup> In fact, if cancer is a disorder of somatic-cell genetics resulting in uncontrolled growth, it is clear that gene therapy will have a definite impact on cancer therapy in the future. Perhaps there is no area in which gene-marking studies are more relevant for understanding disease biology.

**Gene-Marking Studies.** The first human gene-marking study attempted to better define the in vivo distribution and survival of TIL through retroviral-mediated gene transfer.<sup>45</sup> In the first reported cases, it was demonstrated that the marked cells can persist in the circulation for at least 21 days and, in one patient, for up to 189 days. Gene-marked TIL was recovered from tumor deposits up to 64 days after administration. Five additional patients have subsequently been treated, with similar results.<sup>28,44</sup>

The persistence of the gene-marked TIL has prompted the use of gene-marked or "reporter" cells to better understand other aspects of cancer biology (Table 3).

Several institutions, including one in France, are using NeoR-transduced TIL in patients with melanoma or renal-cell cancer. While these studies demonstrate an institution's ability to perform gene transfer experiments, some investigators have expanded on the original concept. For example, Lotze et al.<sup>55</sup> at the University of Pittsburgh are using a combination of both interleukin-2 (IL-2) and interleukin-4 (IL-4) to cultivate TIL. They will then determine if this combination alters the transduction efficiency and tumor infiltration. Investigators at the University of California at Los Angeles (UCLA) are marking peripheral-blood leukocytes with one NeoR vector (G1Na) and TIL with a slightly different NeoR vector (LNL6) to quantify tumor infiltration by these two cell types.

Autologous BMT is a possible curative modality for several malignancies. Despite bone marrow removal during remission, marrow purging, and marrow purification, many patients relapse after the transplant. To unravel the complexities of transplantation and to determine the source of relapse in patients treated with autologous BMT for hematological malignancies, such as acute or chronic myelogenous leukemia, or for solid tumors, such as neuroblastoma, investigators will use the NeoR-carrying vector to mark bone marrow cells.<sup>80,82</sup> With increased transduction efficiency and very sensitive detection techniques such as PCR, it is estimated that as few as 100 tumor cells need to be marked in order to detect them in the circulation following transplant.<sup>82</sup> Demonstration that marrow can be the source of relapse in these patients might lead to more efficient purging of the marrow before it is infused, whereas demonstration that the relapse comes from circulating peripheral cells might lead to more rigorous ablative therapy. Data are available from two patients with acute myelogenous leukemia treated at St. Jude Children's Research Hospital (Memphis, TN) who have relapsed after BMT and the NeoR gene marker has been identified in the resurgent blast cells, suggesting that bone marrow obtained during remission can contribute to disease relapse.<sup>82</sup> As a result, a new gene-marking study to compare two different marrow purging techniques was developed and has recently been approved.

Peripheral-blood stem cells are used alone or in conjunction with bone marrow to hasten hematological recovery following bone-marrow ablative chemotherapy. While the clinical effects of using peripheral-blood stem cells are well documented, no studies have definitively determined that these cells are an extra source of marrow reconstitution. Studies suggest that peripheral-blood stem cells can be transduced with the NeoR gene vector.<sup>88</sup> In a series of studies conducted at NIH, patients with chronic myelogenous leukemia, multiple myeloma, or breast cancer will have both their peripheral-blood stem cells and their bone marrow removed and transduced with the NeoR gene before receiving high-dose antineoplastic therapy. Similar to the NeoR marking studies at UCLA, the peripheral-blood stem cells will be marked with a

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slightly different NeoR vector than the bone marrow. This will enable the investigators to determine the relative contributions of each of the cell types. They will also be able to determine the source of relapse, should that occur, in each of these studies.

Additional studies at NIH are planned using the multidrug resistance gene-1 (MDR1) to mark peripheral-blood stem cells and bone marrow cells from patients receiving BMT for breast cancer. Murine studies have demonstrated that transduction of the MDR1 gene into bone marrow can confer resistance to paclitaxel and provide *in vivo* enrichment of transduced cells.<sup>89</sup> If the clinical study is successful, it may be possible to use a dominant selectable marker, whereby researchers can amplify cell clones *in vivo* that have been cotransduced with the MDR1 gene and an additional therapeutic gene.

**Gene Therapy Studies.** Many studies are under way involving gene therapy for treatment of malignancies. Novel approaches to treating cancer with gene transfer techniques are summarized in Table 5.<sup>80,82</sup>

Adding new genes to cells to give them new functions can have many applications in cancer therapy. Early work toward developing adoptive immunotherapy with lymphokine-activated killer (LAK) cells and then TIL has provided the foundation for several ongoing human gene therapy trials.<sup>26,44,45</sup> Like a sophisticated drug-delivery system, TIL are being used to deliver foreign therapeutic genes directly to tumors. In the first trial, the gene for tumor necrosis factor (TNF) is inserted into TIL (TNF-TIL). In previous clinical studies, TNF was an ineffective antitumor agent, perhaps because

dose-limiting hypotension prevented attainment of sufficient cytotoxic concentrations *in vivo*. However, animal studies using TNF-TIL have demonstrated that localized TNF concentrations of up to 1000 µg/g of tissue can be achieved (concentrations above 400 µg/g of tissue can result in tumor necrosis). Initially, the RAC allowed administration of TNF-TIL alone; when safety was demonstrated, the investigators were allowed to add systemic aldesleukin in increasing dosages.

Data reported from five patients treated with TNF-TIL alone and TNF-TIL plus aldesleukin revealed that both therapies were safe to give to patients and neither produced TNF-related hypotension as seen with TNF alone. When a tumor-site biopsy obtained from a responding patient was examined, the tumor regression seen was consistent with classic TNF-induced coagulative necrosis and not the lymphocyte infiltration seen with TIL therapy.<sup>28,44</sup> This suggests that a local TNF effect was responsible for tumor regression. However, the merits of this trial have recently been questioned, as it appears that the transduced TIL are unable to uniformly express TNF at the projected amounts necessary for tumor regression and the TNF transduced TIL may have a different distribution pattern compared with that previously demonstrated for untransduced TIL.<sup>90</sup>

Other possible genetic modifications of TIL to improve antitumor activity include the introduction of (1) other immunomodulatory cytokines or proteins, such as α-interferon or γ-interferon, interleukin-1, interleukin-6 (IL-6), or interleukin-7; (2) Fc receptor, which mediates antibody-dependent cellular cytotoxicity; (3) chimeric T-cell receptors, which have altered T-cell specificity; and (4) the IL-2 receptor, which makes TIL more sensitive to IL-2.<sup>28,44,84</sup>

Because of the difficulties with gene transfer into T lymphocytes, researchers have been investigating cytokine expression in tumor cells.<sup>53,84</sup> Cytokine genes inserted into tumor cells can make the tumor cells more immunogenic by increasing their recognition by host defenses.<sup>28,44,83,84</sup> These observations, made in murine models using genes for interleukin-1, IL-2, IL-4, IL-6, TNF, γ-interferon, or granulocyte-macrophage colony-stimulating factor (GM-CSF), led to several clinical trials in which patients were immunized with their own tumor cells after those cells were modified by *ex vivo* retroviral-mediated gene transfer.

In Rosenberg's first trial, tumor cells were modified by insertion of the TNF or IL-2 gene.<sup>28,44</sup> Small quantities (less than 2% of the estimated total tumor burden) of these gene-modified tumor cells were then injected subcutaneously into the thigh and intradermally into nearby sites. Three weeks later, the draining lymph nodes were removed and grown in IL-2. The resulting TIL were then given back to the patient with systemic aldesleukin doses, just as in previous TIL protocols.<sup>28,44</sup> Preliminary observations reported from five patients who had completed the first part of the study, in which TNF gene was inserted into tumor cells, suggest that a marked immunologic response can be mounted.

**Table 5.**  
**Possible Genetic Therapies for Cancer<sup>a</sup>**

Gene addition
To lymphocytes to produce active cytokine within tumor (e.g., TNF or IL-2 gene added to TIL)
To tumor cells to make them more immunogenic (e.g., TNF, IL-2, or HLA-B7)
To produce new cytotoxic or cytostatic product within tumor (e.g., VDEPT or sensitivity genes)
To produce protein product with new function (e.g., bone marrow protection by CSF or MDR1 expression)
To introduce tumor suppressor genes (e.g., p53)
Down-regulation of specific gene expression using informational drugs
Antisense oligonucleotides targeted at mRNA
Specific ribozymes targeted at mRNA
Oligonucleotide-triplex DNA formation targeted at DNA
Oligonucleotides targeted at RNA polymerase or transcription factors
Proteases targeted at proteins
Gene replacement for mutant oncogenes or tumor suppressor genes
Homologous recombination
Excision
Gene-function blocker

<sup>a</sup>TNF = tumor necrosis factor, IL-2 = interleukin-2, TIL = tumor-infiltrating lymphocytes, HLA = human lymphocyte antigen, VDEPT = virally directed enzyme prodrug therapy, CSF = colony-stimulating factor, MDR1 = multidrug resistance gene-1, mRNA = messenger RNA. (Adapted from reference 78, with permission.)

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Taking a different approach for increasing tumor immunogenicity, investigators at the University of Michigan will introduce the gene for human lymphocyte antigen (HLA)-B7 directly into tumors in vivo by using DNA-liposome complexes.<sup>24</sup> It has been demonstrated in mice that when a cytotoxic T-cell response is induced, the gene product stimulates immunity to other antigens present on unmodified tumor cells and tumor regression occurs. The University of Michigan trial is one of the first gene therapy studies in which the gene transfection occurs directly in vivo rather than by processing the cells ex vivo and returning them to the patient.

Immunizing patients with their own gene-modified tumor cells can also be considered the first step toward developing a tumor vaccine. While the ongoing studies might provide a method for individualized vaccination, it is envisioned that one day the genes coding for tumor-associated antigens could be inserted into a virus, such as vaccinia, that might serve as a primary or secondary cancer prevention method. Studies are ongoing to identify these tumor-associated antigens and to determine if several tumor types share common antigens.<sup>24,44</sup>

Virally directed enzyme prodrug therapy, sometimes referred to as suicide or sensitivity gene therapy, is a manipulation that takes advantage of proliferative, transcriptional, or enzymatic differences between tumor and normal cells to cause preferential tumor cell death.<sup>40,42,91,92</sup> For one technique, a gene for a drug-activating enzyme (e.g., herpes simplex virus thymidine kinase [HSV-TK]) is inserted into a tumor cell either in vivo or ex vivo.<sup>40,42,91,93,94</sup> The actively dividing tumor cell then expresses the protein product of that gene (e.g., thymidine kinase). Upon uptake of a drug that is activated by this enzyme (e.g., acyclovir or ganciclovir sodium), the tumor cell dies. To restrict gene expression of the drug-activating enzyme to tumor cells, a gene can be constructed that is composed of a tissue-specific transcriptional regulatory sequence (e.g., promoter sequence) adjacent to the sequence coding for the drug-activating enzyme.<sup>91</sup> Thus, if the promoter sequence for  $\alpha$ -fetoprotein (hepatoma), prostate-specific antigen (prostate cancer), or carcinoembryonic antigen (colorectal or lung cancer) is adjacent to the gene encoding for a drug-activating enzyme, then normal tumor synthesis of the protein or antigen will result in synthesis of an enzyme that will activate the subsequently administered cytotoxic agent.

A trial recently initiated at NIH in patients with either primary brain tumors or brain metastases secondary to lung cancer, breast cancer, malignant melanoma, or renal cell carcinoma is introducing the gene for HSV-TK directly in situ by using retroviral-mediated gene transfer.<sup>93</sup> It has been demonstrated that only actively dividing cells integrate retroviral vectors and express HSV-TK. In animals, normal neural cells, which have lost their capacity to divide, are spared.<sup>92,94</sup> A trial using

the HSV-TK suicide-virus gene concept has also been proposed for patients with ovarian cancer.

Gene addition can also be used to provide an indirect therapeutic advantage by manipulating normal cells to make them more resistant to cytotoxic chemotherapy. As already described, peripheral-blood stem cell and bone marrow-marking studies using the MDR1 gene are planned.<sup>95</sup> Preliminary evidence suggests that cell clones expressing MDR1 are resistant to the effects of cytotoxic drugs and can grow after treatment with these drugs. Patients in these studies will receive either paclitaxel or vinblastine sulfate if they have progressive disease following the bone marrow transplant. If the transplanted cells continue to express MDR1 and the population expands, the investigators expect to see progressively shorter and shorter nadir leukocyte counts with subsequent paclitaxel or vinblastine sulfate treatments. Similarly, it is possible that hematopoietic protective effects can be achieved by transducing hematopoietic stem cells with genes coding for protective cytokines such as GM-CSF, granulocyte colony-stimulating factor, interleukin-3, or stem cell factor.<sup>90</sup> Also, as many tumors result in part from inactive or missing tumor suppressor genes, gene addition therapy might be able to provide these missing protective genes.<sup>93,97</sup>

It is also possible to inhibit specific tumor growth functions at the genetic level by down-regulating the expression of oncogenes by introducing "informational" drugs or compounds with antisense relationships to specific nucleotide sequences. These compounds include oligonucleotides (which can block mRNA, DNA, RNA polymerase, or transcription factor activity), ribozymes (which can cut RNA at a specific target site), or proteases (which can inactivate several essential tumor proteins).<sup>80,82</sup>

Human tumors can result from overexpression of oncogenes, failure to express certain tumor suppressor genes, or point mutations that activate transforming functions.<sup>95,96,98</sup> Another strategy for gene therapy manipulation would be to replace mutant genes with a normal copy of the gene by homologous recombination or by molecularly excising the mutant gene and replacing it with a normal copy.<sup>80,82</sup> Homologous recombination has been shown to work well in vitro, but it is currently too inefficient to use in clinical trials.<sup>80</sup>

In the first attempt to target the genetic basis for cancer, researchers at The University of Texas M. D. Anderson Cancer Center (Houston) will focus on two mutations associated with lung cancer: the *K-ras* oncogene, which is present in 30-40% of lung adenocarcinomas, and the p53 tumor suppressor gene, which is deleted in 50-70% of all lung cancers.<sup>95,97,99</sup> For patients with *K-ras*, the investigators will directly inject a retroviral vector supernatant containing a construct with the mirror image of the *K-ras* mRNA into the tumor. This antisense *K-ras* should prevent the decoding of the *K-ras* message and should stop uncontrolled tumor proliferation. For patients with either a p53 gene mutation or deletion, a correct copy of the p53 gene will be

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transfected directly into the tumor. In both cases a murine retrovirus will be used to insert the gene, and animal studies have demonstrated that approximately 50% of tumor cells will integrate this new gene. This is the first attempt to individualize gene therapy based on a patient's specific gene mutation.

While many of the clinical trials using gene transfer techniques to better understand cancer biology or to treat cancer are still in very primitive stages, many more studies will soon be initiated. As we gain more insight into the processes behind the interactions of DNA, RNA, and proteins during transcription and as more oncogenes, tumor suppressor genes, and tumor-associated proteins are discovered, it is likely that we will be able to target cancer treatment directly at the genetic level.

**Nongenetic, Nonmalignant Diseases.** Many nongenetic, nonmalignant diseases are targeted for gene therapy approaches. Among the most actively pursued areas are HIV infection and cardiovascular conditions. Gene therapy for other diseases are sure to be explored shortly.

Perhaps more is known about the molecular biology surrounding HIV infection and perpetuation than any other infectious disease. Gene therapy strategies will take advantage of this knowledge by either modifying cells to protect them from infection or by targeting transcriptional or translational inhibitors of viral protein production.<sup>46,101-102</sup> In the first approved HIV-related gene therapy trial, researchers at the Fred Hutchinson Cancer Center (Seattle, WA) are using CD8<sup>+</sup>, HIV-specific, cytolytic T cells transduced with both a marker gene and a suicide gene for patients with HIV-related lymphoma undergoing allogeneic BMT. It is hoped that the high-dose antineoplastic therapy, total body irradiation, and these cytolytic T cells will eradicate all HIV-infected cells in the patient and prevent infection of the healthy marrow, and that the transplanted marrow will repopulate the hematologic lines with HIV-free cells. The gene encoding hygromycin phosphotransferase is being used to mark the cytolytic T cells to follow cell survival. Since these cytotoxic T lymphocytes are known to produce central nervous system toxicity and lymphocytic alveolitis, transduction of the gene for HSV-TK will allow the investigators to ablate the transduced cells with acyclovir or ganciclovir sodium should untoward toxicity occur. In another trial, researchers will genetically alter fibroblasts to manufacture gp160, a protein that is part of the HIV protective envelope.<sup>102</sup>

Investigators have also described techniques to construct retroviral vectors capable of expressing soluble CD4, the cell-surface receptor for HIV binding, thus opening the possibility for the patient's own cells to manufacture this protein to block HIV infection of surrounding cells.<sup>46,100</sup> Researchers are also actively constructing decoy molecules directed at the major HIV-genomic elements, such as reverse transcriptase, transactivating factor (TAT), the TAT-binding area (TAR), or REV (a viral protein required for viral

mRNA translation).<sup>100,102</sup> Some researchers have proposed to use the HIV-1 virus itself as a retroviral vector delivery vehicle for transducing anti-HIV genes into target cells, as it has been demonstrated that this vector can enter cells in the same manner that HIV can.<sup>102</sup>

Faraji-Shadan et al.<sup>101</sup> have described a novel technique for intracellular immunization against HIV infection by using antibodies created by immunizing naive B cells with reverse transcriptase or TAT. These selected antibodies, when introduced into the cytoplasm and nucleus, would interfere with viral replication. This technique may be applicable to other retroviral diseases such as adult T-cell lymphoma associated with human T-cell lymphotropic virus type I.<sup>101</sup>

Despite what is known about HIV infection and the overwhelming enthusiasm to use gene therapy to prevent or treat this devastating disease, there are still many unanswered questions and hurdles to overcome. For instance, the critical level of T-cell protection that is needed to confer overall patient protection from the clinical consequences of HIV infection is not known. Nonetheless, HIV infection will continue to be actively pursued for gene therapy.

More people die from cardiovascular disorders than any other disease, making it a natural target area for gene therapy. Vascular grafting is commonly used to alleviate the symptoms associated with vascular blockages due to coronary artery or peripheral vascular disease. In other cases, vascular stents are used to maintain vein patency. While these procedures are initially effective, more than 30% fail because the grafts clot. Researchers are currently investigating methods to engineer endothelial cells to secrete anticoagulating compounds, such as TPA.<sup>103,104,105</sup> Preliminary data suggest that these genes can function in vivo.<sup>104,105</sup> These genetically altered endothelial cells can then be implanted in the graft or stent to prevent clotting. This represents yet another application of gene therapy, that is, to prevent a complication associated with a surgical procedure.

**Obstacles To Overcome.** Will we be able to cure or treat all diseases with gene therapy? There are still many obstacles to overcome. For instance, progress is slow in developing injectable vectors to simplify foreign gene administration. Perhaps the biggest problem to overcome will be engineering the target cells to be able to regulate the gene expression according to physiologic needs. With diabetes mellitus, for example, it might be possible to engineer cells to secrete a constant amount of insulin and prevent the effects of absolute insulin deficiency.<sup>106</sup> But getting these cells to secrete insulin in response to the individual's diet just as the normal pancreas does is not close to being achieved yet.

## Conclusion

The initiation of clinical gene therapy trials has heralded a new age in medicine. From the time the first

human received foreign genes just over four years ago, nearly 40 additional clinical trials have been approved and more than 110 patients have been entered in gene therapy studies. As the human genome is deciphered and more pathologic genes are identified, gene therapy trials will surely expand at a logarithmic rate. Pharmacists should become knowledgeable about gene transfer techniques and possible clinical applications of gene therapy to keep abreast of the newest trends in medicine.

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COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

IN THE MATTER OF: Australian  
Patent Application 696764  
(73941/94). In the name of:  
Human Genome Sciences Inc.

- and -

IN THE MATTER OF: Opposition  
thereto by Ludwig Institute for Cancer  
Research, under Section 59 of the  
Patents Act.

Annexure GBC-14

This is **Annexure GBC-14** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
\_\_\_\_\_  
**Gary Baxter Cox**

WITNESS:

  
\_\_\_\_\_  
Patent Attorney

PEYTON K. HOO

08.93811553

secreted by transfected COS or Chinese hamster ovary cells begins at a glycine residue at position 21; the non-glycosylated form has a molecular mass of 12,397 by electrospray mass spectrometry, close to the predicted *M<sub>r</sub>* of 12,399, although it migrates as a 9K protein on SDS-PAGE (Fig. 1C).

The production by monocytes of inflammatory cytokines such as IL-1, IL-6 and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a crucial initiating event in a number of infectious and inflammatory pathologies. We have found that IL-13 strongly inhibits IL-6 secretion induced by bacterial lipopolysaccharide (LPS) in peripheral blood mononuclear cells (PBMC) (Fig. 3a). IL-13 would appear to be acting directly on monocytes because an inhibition of IL-6 mRNA accumulation is observed rapidly (within 4 hours) in cultures of PBMC enriched in monocytes by adherence to tissue culture dishes (Fig. 3b). A marked inhibition is also seen for other inflammatory monokine mRNAs (IL-1 $\beta$ , TNF- $\alpha$ , IL-8, gro- $\beta$ ) in LPS-treated monocytes in the presence of IL-13 (Fig. 3b, and other data not shown). The action of IL-13 would thus seem to be a generalized block on inflammatory monokine synthesis, a property shared with the other Th2 lymphokines IL-4 and IL-10 (ref. 13). IL-13 and IL-4 show similar levels of inhibition of IL-6 synthesis (Fig. 3a). IL-13 also inhibits production of human immunodeficiency virus (HIV) by tissue-culture differentiated macrophages<sup>14</sup>, contrasting with the stimulatory effects of macrophage-activating cytokines such as IL-3 and GM-CSF on HIV production that have been reported in comparable conditions<sup>15</sup>.

The production of IFN- $\gamma$  by large granular lymphocytes (LGL) may direct subsequent immune responses, leading to macrophage activation and to a 'Th1-type' cellular immune response<sup>16</sup>. The major cytokine influencing this production of IFN- $\gamma$  is IL-2 (ref. 17). We have found that IL-13 has a small, direct effect on IFN- $\gamma$  synthesis by LGL, and synergizes with both suboptimal and optimal doses of IL-2 (Fig. 3c). In this respect it resembles IL-12 (ref. 17), rather than IL-4, which strongly inhibits IFN- $\gamma$  synthesis by these cells (Fig. 3c).

IL-13 also affects B lymphocytes, increasing their proliferation and the expression of the CD23 surface antigen (P. Carayon and T. DeFrance, personal communication). IL-13 is thus a highly pleiotropic cytokine. In its anti-inflammatory effects on monocytes and its stimulation of the humoral response through B lymphocytes, IL-13 contributes to the 'Th2-type' response together with IL-4 and IL-10 (refs 18, 19). In, however, its effects on IFN- $\gamma$  synthesis, it might be expected to promote a 'Th1-type' cellular immune response<sup>16,20</sup>. A full understanding of the cytokine network in different pathological situations now needs to take into account the activities of IL-13.

The anti-inflammatory function of IL-13 may be crucial in clinical inflammation, for example in septic shock<sup>21</sup> or rheumatoid arthritis<sup>22</sup>. Its activity on LGL may be clinically interesting in that, unlike IL-4, it does not decrease and can even increase the IL-2-induced lymphokine-activated killer activity of these cells (our unpublished results). As IL-13 also inhibits HIV replication *in vitro*<sup>14</sup>, and systemic immunity to parental tumour cells can be induced by IL-13-secreting tumour cells *in vivo* (D. Fradelizi, personal communication), IL-13 would appear to represent a potentially important new member of the therapeutic cytokine arsenal. □

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## Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy

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CYSTIC fibrosis (CF) is a lethal inherited disorder affecting about 1 in 2,000 Caucasians. The major cause of morbidity is permanent lung damage resulting from ion transport abnormalities in airway epithelia that lead to mucus accumulation and bacterial colonization. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene<sup>1</sup> that encodes a cyclic-AMP-regulated chloride channel<sup>2,3</sup>. Cyclic-AMP-regulated chloride conductances are altered in airway epithelia from CF patients<sup>4-6</sup>, suggesting that the functional expression of CFTR in the airways of CF patients may be a strategy for treatment. Transgenic mice<sup>7-9</sup> with a disrupted *cftr* gene are appropriate for testing gene therapy protocols. Here we report the use of liposomes to deliver a CFTR expression plasmid to epithelia of the airway and to alveoli deep in the lung, leading to the correction of the ion conductance defects found in the trachea of transgenic (*cf/cf*) mice. These studies illustrate the feasibility of gene therapy for the pulmonary aspects of CF in humans.

Plasmid DNA complexed with cationic liposomes can be successfully delivered and expressed in airway epithelia of rodents<sup>10,11</sup>. A suitable plasmid for expressing CFTR protein was constructed in the vector pREP8 (see legend to Fig. 1). In this plasmid, pREP8-CFTR, the human CFTR complementary DNA is under transcriptional control of the constitutive Rous sarcoma virus (RSV) 3' long terminal repeat (LTR) promoter, known to be active in nonproliferating airway epithelial cells<sup>12</sup>. To show that pREP8-CFTR expresses CFTR protein after

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transfected was in western blot. CFTR was not detected in cAMP from cells of this report. transfection protein: RN. CFTR cells b messenger: intestinal: control mouse probes *cftr* pr mRNA antisense with m. This does not. After mice of CFTR hybrid: cells us

FIG. 1 B. HeLa cells of HeLa cells with pREP8-CFTR. Cells are indicated crosses. Time course plasmid point at v as the percentage. METHODS (nucleotide under transfection). pREP8-CFTR sequence at nucleotides (CCACCA) transfection. Tissue culture with 10% in each well. Lipofectin a further extraction cells were containing inhibitors (310 µg/ml methylsulphonyl fluoride) removed from pelleted cells dissolved SDS-polyacrylamide gel electrophoresis (SDS-PAGE) anti-CFTR: once (ECL cells were perature in 11 mM glu



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transfection, plasmid DNA complexed with cationic liposomes was introduced into HeLa cells and CFTR protein detected by western blotting (Fig. 1a). To ascertain whether the expressed CFTR protein was functional, cAMP-stimulated iodide efflux was measured in transfected cells (Fig. 1b). In HeLa cells transfected with pREP8-CFTR, iodide efflux was stimulated by a cAMP-agonist cocktail. The cocktail did not stimulate efflux from cells transfected with the vector pREP8. The characteristics of this cAMP-stimulated anion efflux were similar to those reported previously for CFTR-expressing cells<sup>12,13</sup>. Thus, cells transfected with pREP8-CFTR express functional CFTR protein.

RNA *in situ* hybridization was used to demonstrate that the CFTR expression plasmid can be delivered to airway epithelial cells by liposome-mediated transfection *in vivo*. Because CFTR messenger RNA is expressed at high levels in human and rodent intestinal crypts<sup>14-16</sup>, mouse intestinal sections were used as controls to demonstrate probe specificity. No hybridization to mouse intestine was detected with either of two human CFTR probes whereas, in consecutive sections, the mouse antisense *cfr* probe (but not the sense probe) detected abundant *cfr* mRNA in the crypts (data not shown). Additionally, neither the antisense nor the sense *hisD* vector control probes hybridized with mouse intestinal mRNA, as expected (data not shown). This demonstrates that the human CFTR and vector *hisD* probes do not cross-hybridize with mouse *cfr* mRNA.

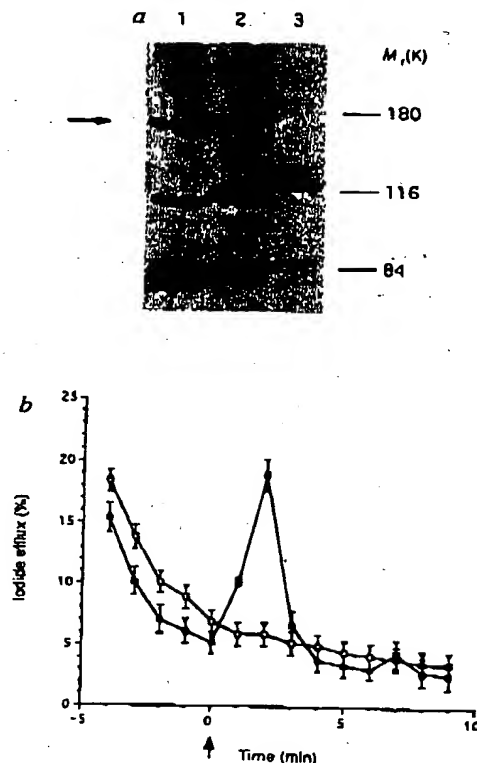
After transfection of pREP8-CFTR DNA into the airways of mice of 20-28 days old, sequences corresponding to human CFTR were detected by *in situ* hybridization (Fig. 2). Strong hybridization signals were observed in isolated groups of airway cells using both the human CFTR probe (Fig. 2a-c) and the

*hisD* vector-specific probe (d-f). In a series of consecutive sections the hybridization signals observed with the human CFTR and *hisD* probes colocalized to the same airways and airspaces (Fig. 2a-f). No hybridization was detected with the mouse *cfr* probe. This provides strong evidence that the hybridization signals obtained are highly specific and due to the transfected plasmid. Hybridization of these same probes to lung sections from untransfected animals served as a negative control against nonspecific hybridization; neither the human CFTR probe (g-i) nor the *hisD* probe (j-l) hybridized to any mRNAs in the lungs of untransfected mice. Hybridization signals were obtained with both the sense and antisense probes (Fig. 2a-f). Normally the antisense probe is used to detect mRNA whereas the sense probe serves as a negative control. Following transfection, however, both the sense and antisense probes would be expected to recognize vector DNA. The stronger signal observed with the antisense probe indicates transcription. This was seen for the *hisD* gene which is transcribed from a vector promoter. In most hybridizing cells, the signal obtained with the antisense human CFTR probe (Fig. 2b) was also greater than that obtained with the sense probe (c), implying that human CFTR mRNA is expressed following transfection.

The data in Fig. 3 show hybridization to sections through different regions of the lungs of a mouse which had been transfected with pREP8-CFTR. No expression of endogenous mouse *cfr* mRNA was detected in any region of the lung (data not shown), consistent with previous studies showing low-level *cfr* expression in rodent lung<sup>14</sup>, and with detailed studies of these transgenic animals (A.E.O.T. *et al.*, manuscript in preparation). This shows that the transfection protocol does not induce expression of endogenous mouse *cfr* mRNA. Human CFTR

**FIG. 1** Expression of functional CFTR protein from plasmid pREP8-CFTR in HeLa cells. **a**, Western blot confirming expression of CFTR after transfection of HeLa cells using Lipofectin. Lanes 1, HT29 cells; 2, HeLa cells transfected with pREP8-CFTR; 3, HeLa cells transfected with the vector pREP8. The HT29 cells served as a positive control for CFTR expression and migration<sup>25,26</sup> indicated by the arrow. The ~115 and 85K bands are due to nonspecific cross reactions of the antibody<sup>25</sup>.  $M_r \times 1,000$  of markers is indicated. **b**, Time course of iodide efflux from HeLa cells. Cells were transfected with plasmid pREP8-CFTR (■) or the vector pREP8 (□). The arrow indicates the point at which a cAMP-agonist cocktail was added. The data are displayed as the mean of three individual experiments ( $\pm$ s.e.m.), expressed as a percentage of the total efflux.

**METHODS.** Human CFTR cDNA encoding the entire CFTR coding sequence<sup>1</sup> (nucleotides 133-4,620) was inserted into the plasmid pREP8 (Invitrogen), under transcriptional control of the RSV 3' LTR promoter, to create plasmid pREP8-CFTR. The cDNA incorporated three minor changes from the published sequence (C to G at nucleotide 136<sup>13</sup>; T to C at nucleotide 936<sup>27</sup>; A to C at nucleotide 1990<sup>28</sup>), and included a Kozak translation initiation sequence<sup>20</sup> (CCACCATG) immediately 5' to the translation initiation codon. For plasmid transfection,  $1 \times 10^6$  HeLa cells were seeded into each well of 35-mm, 6-well tissue culture dishes in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum, and incubated at 37 °C. After 24 h growth, cells in each well were transfected with 8  $\mu$ g plasmid DNA mixed with 13  $\mu$ g Lipofectin (Gibco BRL) and diluted to 3 ml in Optimem 1 (Gibco BRL). After a further 24-48-h incubation at 37 °C, cells were either collected for protein extraction or used for anion efflux measurements. For protein extraction, cells were washed five times with ice-cold PBS and collected into a buffer containing 10 mM Tris-Cl pH 8.0, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, and the protease inhibitors: antipain (50  $\mu$ g ml<sup>-1</sup>), aprotinin (10  $\mu$ g ml<sup>-1</sup>), benzamide (310  $\mu$ g ml<sup>-1</sup>), leupeptin (5  $\mu$ g ml<sup>-1</sup>), pepstatin A (5  $\mu$ g ml<sup>-1</sup>) and phenylmethylsulphonyl fluoride (175  $\mu$ g ml<sup>-1</sup>). Cells were lysed by repeated passage through a 19-gauge needle. Cellular and nuclear debris were removed from the lysate by a 5-min centrifugation at 300g and membranes pelleted by a 30-min centrifugation at 100,000g. The membrane pellet was dissolved in 2.5% Triton X-100 and separated by electrophoresis on a 6% SDS-polyacrylamide gel. CFTR was detected by western blotting after transfer to a Hybond C-super membrane (Amersham) using the well characterized anti-CFTR antiserum 181<sup>25</sup>. Immunodetection was by enhanced chemiluminescence (ECL; Amersham). To measure cAMP-stimulated efflux, the transfected cells were preloaded with iodide by incubation for 40 min at room temperature in 3 ml loading buffer (136 mM NaI, 3 mM KNO<sub>3</sub>, 2 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 11 mM glucose, 20 mM HEPES, pH 7.4). Extracellular NaI was removed by



6 × 1 ml rinses in efflux buffer (loading buffer with 136 mM NaNO<sub>3</sub> replacing the NaI). Cells were then washed with 1 ml efflux buffer for 1 min using a sample-replace procedure. After the fifth 1-min sample (designated time 0), cAMP-agonists (1 mM 3-isobutyl-1-methylxanthine (IBMX), 200  $\mu$ M dibutyryl-cAMP, 10  $\mu$ M forskolin, dissolved in DMSO) were included in the efflux buffer. The concentration of iodide in each 1-ml aliquot was determined using an iodide-specific electrode (HNU systems).

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expression was seen in the airways of three out of four animals transfected with pREP8-CFTR and, in at least one transfected animal, human *CFTR* sequences were detected in all five lobes of the lung. Positive cells were detected in large and small airways (Fig. 2a-d), and in cells lining the air spaces of the more distal regions of the lung (e-j). It appeared to be the surface epithelial cells of the airways that had been transfected. Colocalization of the *CFTR* signal with the *hisD* probe (Fig. 2), confirmed that the signal was a consequence of transfection.

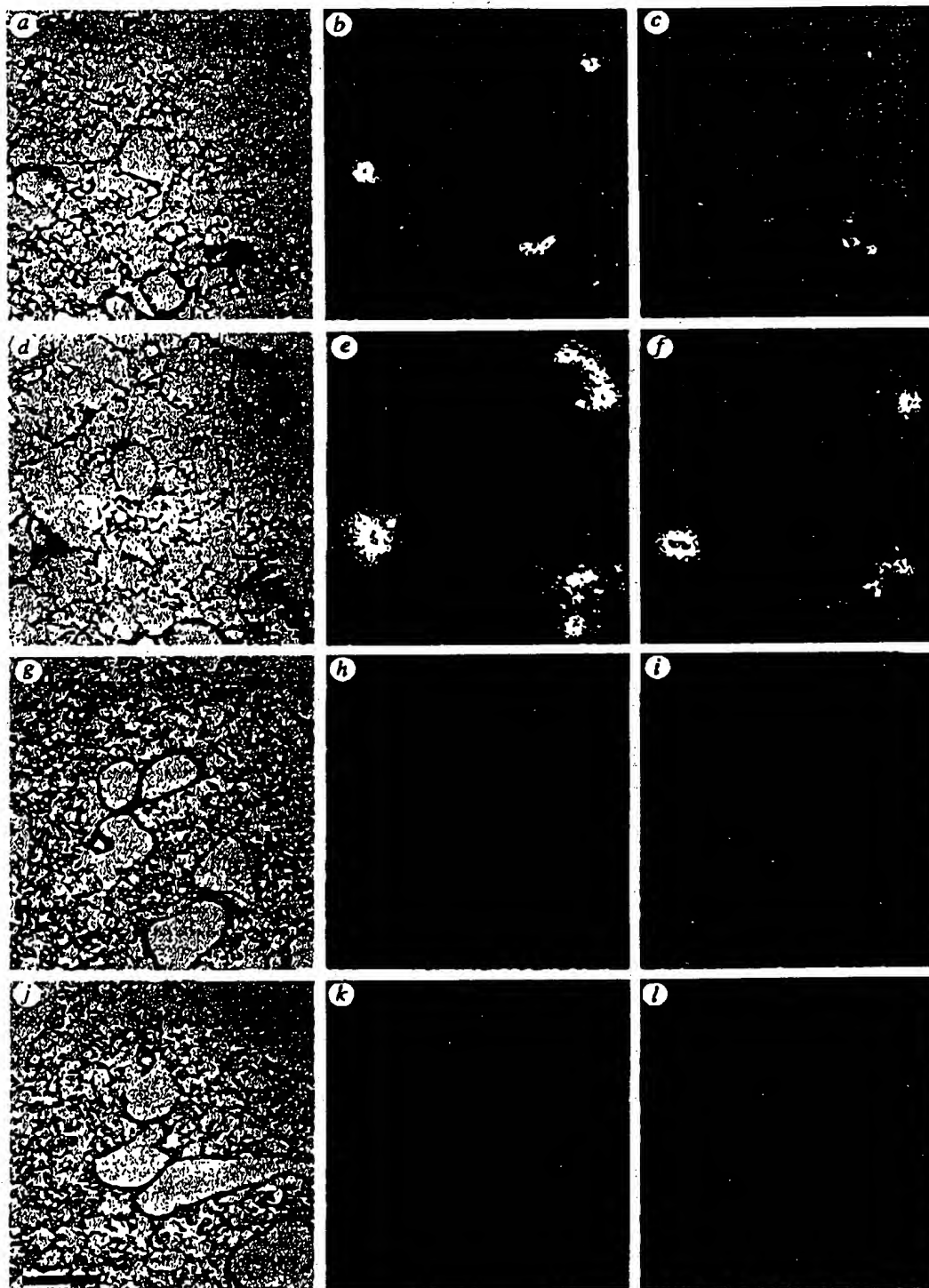
**FIG. 2** Detection of human *CFTR* by *in situ* hybridization in mouse airways following *in vivo* transfection. a-f, Data obtained for a mouse transfected with pREP8-CFTR; g-i, controls for an untransfected mouse. The probes used were against human *CFTR* exons 1-6 (a-c and g-i) or the *hisD* vector sequences (d-f and j-l). For each example, three panels are shown: (1) a brightfield view of a section hybridized with the antisense probe, to illustrate tissue morphology (a, d, g, j); (2) a darkfield view of the same section (b, e, h, k); (3) a darkfield view of an adjacent section probed with the control sense probe (c, f, i, l). Scale bar, 200  $\mu$ m. Similar results were obtained with several animals.

**METHODS.** Mice were given enough avertin by intraperitoneal injection to induce very light anaesthesia. For transfection, ~100  $\mu$ g plasmid DNA was mixed with 25  $\mu$ g Lipofectin in a total volume of 50  $\mu$ l and administered to mice by tracheal instillation in two loads by insertion of a metal applicator, adapted from a 25-gauge blunted syringe needle, through the mouth and into the trachea to the point where the main bronchial branch off. The animals used weighed between 5 g and 12 g. Four days after transfection, *in situ* hybridization was performed on perfusion-fixed tissue by a modification of the method described by Simmons *et al.*<sup>30</sup>, as described previously<sup>15</sup>. <sup>35</sup>S-labelled RNA probes were synthesized *in vitro* by run-off transcription from plasmid DNA, incorporating [<sup>35</sup>S]UTP. The antisense and sense (control) probes were derived from opposite strands of the same plasmid. The plasmids used for probe generation were as follows. The two human *CFTR* probes, corresponding to nucleotides 62-645 (exons 1-6) and nucleotides 1,977-2,461 (exon 13) (numbering according to ref. 1), have been described previously<sup>16</sup>. The mouse *cftr* probes were derived by reverse transcriptase PCR from mouse testis mRNA and corresponded to nucleotides 305-691 of exons 3-5. The *hisD* vector probe was subcloned from pREP8 and corresponded to nucleotides 3,167-3,851. All probes were cloned into Bluescript vectors (Stratagene). After developing,

Thus, transfection is effective and expression of human *CFTR* throughout the airway was achieved.

To determine whether delivery of *CFTR* cDNA to the airways could correct the ion transport defects apparent in CF, we used a recently developed mouse model<sup>9,17</sup>. These transgenic (*cf/cf*) mice are homozygous for a null mutation in *cftr* and express little or no detectable endogenous *cftr* mRNA (A.E.O.T. *et al.*, manuscript in preparation). *CFTR*-dependent, cAMP-stimulated chloride conductances are greatly reduced in the airways

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**FIG. 3** De mouse air regions of were hyb t ponding t a-d, Expr e-j, expe lung. Son transfecti difference 100  $\mu$ m, hybridizat

sections were counterstained with haematoxylin and eosin and photographed using a Microphot FX microscope.

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and caeca of these mice, compared with normal (+/+) animals, mimicking features of the human disorder<sup>17</sup>. The mice frequently die shortly after birth as a consequence of intestinal blockages. Ion transport in the trachea was measured by voltage clamping at zero potential, using pharmacological agents to eliminate or stimulate various processes (Fig. 4). Measurements were also made with the caecum of the same animal as an internal control. Figure 4A shows a set of typical results, and Fig. 4B a compilation of the data. For each tracheal preparation, three measurements were made: amiloride-sensitive sodium absorption (labelled  $\text{Na}^+$ ), cAMP-stimulated chloride secretion (labelled  $\text{Cl}^-$  cAMP), and  $\text{Ca}^{2+}$ -stimulated chloride secretion (labelled  $\text{Cl}^-$   $\text{Ca}^{2+}$ ). As expected, CFTR-dependent, cAMP-stimulated

chloride secretion was significantly reduced ( $P < 0.01$ ) in both the tracheas and caeca of the *cf/cf* mice compared with the normal (+/+) mice. There was no significant difference in the cAMP-stimulated chloride secretion between untreated and pREP8-transfected normal mice, indicating that transfection itself has no effect on ion transport. Most importantly, transfection of *cf/cf* mice with pREP8-CFTR restored the cAMP-stimulated chloride secretion in the trachea to a level comparable with that of normal (+/+) animals. In sharp contrast, transfection of the *cf/cf* mice with the vector pREP8 had no significant effect on the cAMP-stimulated chloride secretion in the trachea. The caecum of *cf/cf* mice transfected with pREP8-CFTR showed no appreciable cAMP-stimulated chloride secretion

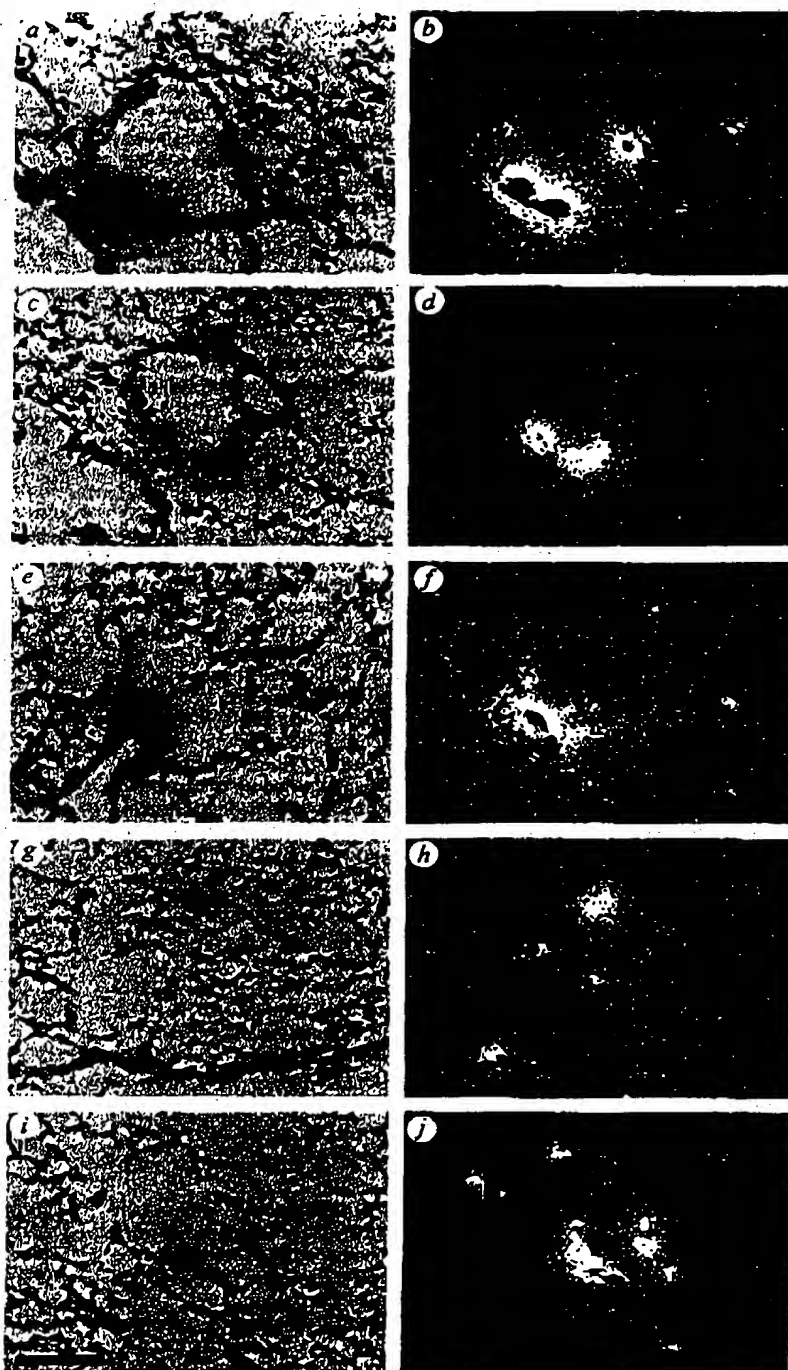
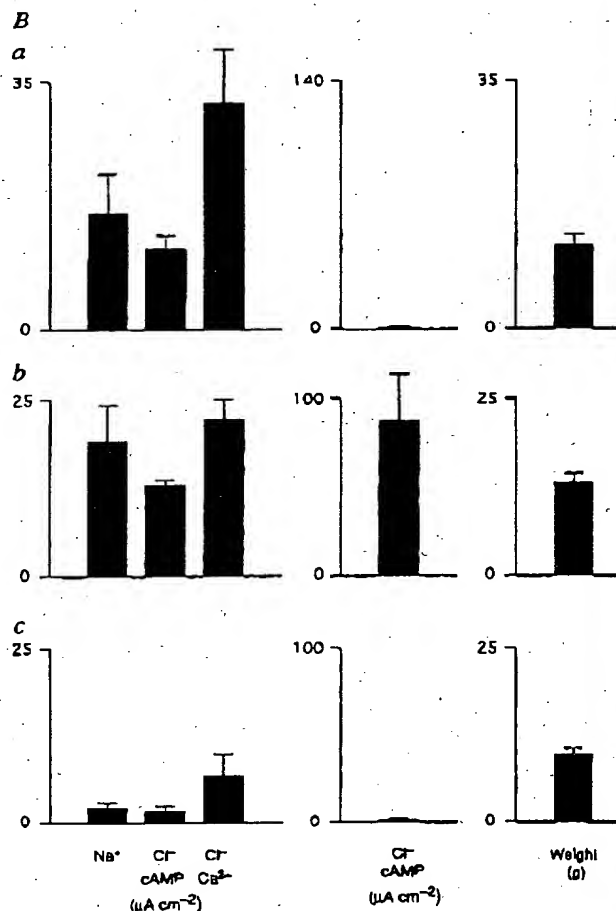
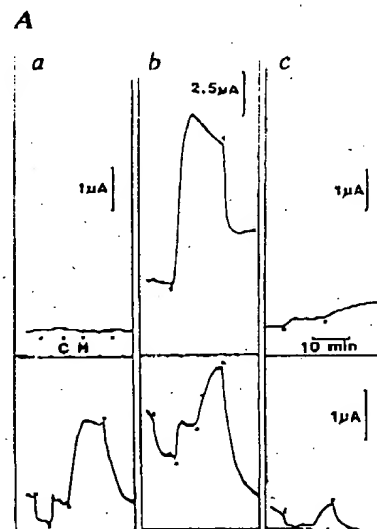


FIG. 3 Detection of human CFTR in different regions of the mouse airway following transfection. Sections from different regions of the airway of a mouse transfected with pREP8-CFTR were hybridized with human antisense CFTR probes corresponding to either exons 1-6 (a-f, i, j) or exon 13 (g and h). a-d Expression of human CFTR in small and large airways; e-j expression in airspaces in the more distal regions of the lung. Some variation was found in the proportion of cells transfected in different animals which probably reflects differences in the amounts of DNA delivered. Scale bar, 100  $\mu\text{m}$ . See legend to Fig. 2 for details of methods and hybridization probes.

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**FIG. 4** Correction of the ion channel defects in the trachea of transgenic (*cf/cf*) mice. **A**, Sample traces showing examples of the data from which panel **B** was compiled. Three paired tracheal/caecal preparations are shown. Upper records show measurements for the caecum and lower records for the trachea of the same animal. **a**, *cf/cf* mouse transfected with pREP8-CFTR; **b**, *+/+* mouse transfected with pREP8; **c**, *cf/cf* mouse transfected with the vector pREP8. Four additions were made to each of the tracheal preparations at the time points indicated by dots. First, amiloride (100  $\mu$ M) was added (apically) to block electrogenic sodium absorption and to ensure subsequent current increases were not due to this activity.  $EC_{50}$  for amiloride is  $\sim 1$   $\mu$ M and 100  $\mu$ M will give essentially 100% inhibition<sup>31</sup>. Second, forskolin (10  $\mu$ M) was added to both sides of the membrane to stimulate adenylate cyclase and activate cAMP-sensitive chloride channels, increasing chloride secretion. Third, the  $Ca^{2+}$  ionophore A23187 (1  $\mu$ M) was added to both sides of the membrane to activate  $Ca^{2+}$ -dependent chloride secretion. The ionophore-induced responses were much slower than those induced by forskolin. Finally, frusemide (1 mM) was added basolaterally to block chloride secretion, confirming the nature of the electrogenic transporting activity. Frusemide (1 mM) inhibits over 90% of  $Cl^{-}$  secretion<sup>17</sup>. Calibrations for the trachea are the same in each panel. Caecal preparations (upper records) received two additions, forskolin (10  $\mu$ M, added to both sides of the membrane) and frusemide (1 mM, added basolaterally). Other additions are specifically labelled: C, carbachol (10  $\mu$ M); H, histamine (10  $\mu$ M). In both the caecum and the trachea the chloride secretory responses were inhibited by frusemide, indicating that they are due to electrogenic chloride secretion from the basolateral to the luminal side of the epithelium. In 8 out of 10 *cf/cf* caeca, frusemide led to a slight increase in short-circuit current (SCC) (c, upper record); this is probably due to a blockage of  $K^{+}$  secretion and is typical of the caecum of *cf/cf* mice<sup>17</sup>. **B**, Compilation of the data. Mice were subjected to three different treatment protocols: **a**, *cf/cf* mice transfected with pREP8-CFTR; **b**, *+/+* mice transfected with the vector pREP8; **c**, *cf/cf* mice transfected with the vector pREP8. Four animals in each group were matched based on a compromise between weight and age. Data were only included when paired airway and caecal measurements could be made for the same animal. The genotypes of the transfected mice, and of the plasmid DNA with which they were transfected, was unknown at the time the measurements were made. For each treatment regime, three sets of data are shown. (1) The left hand columns show SCC measurements for the trachea. Three measurements of SCC changes are presented:  $Na^{+}$ , amiloride-sensitive sodium absorption<sup>32</sup>;  $Cl^{-}$  cAMP, SCC change induced by forskolin, presumed to reflect CFTR function<sup>17,32</sup>;  $Cl^{-}$   $Ca^{2+}$ , SCC change induced by the addition of the calcium ionophore A23187. As about 50% of the basal current in the airways was due to sodium absorption, chloride secretion was measured after the addition of amiloride (100  $\mu$ M) which abolished electrogenic sodium absorption. (2) The central columns show SCC measurements for the caecum. Only cAMP-sensitive chloride secretion ( $Cl^{-}$  cAMP), induced by the addition of forskolin (10  $\mu$ M), was measured. Amiloride was not added because the caecum shows no sodium absorptive current<sup>17,32</sup>. (3) The right-hand columns show the weights of the animals used (mean  $\pm$  s.e.m.). Note: the ion transport characteristics of 4/6 pREP8-CFTR transfected *cf/cf* mice were altered by transfection; the reason for the failure of the other two mice is almost certainly failure in delivery. Nevertheless, the forskolin-sensitive SCC ( $Cl^{-}$  cAMP) in the whole group including the two failures ( $9.2 \pm 2.6$   $\mu A cm^{-2}$ ,  $n=6$ ) was significantly greater ( $P < 0.05$ , Mann and Whitney test) than the value for *cf/cf* mice ( $1.9 \pm 0.5$   $\mu A cm^{-2}$ ,  $n=4$ ). Finally, data for two other groups of animals were obtained although these are not illustrated in the figure. Untreated, wild-type (*+/+*) mice ( $n=5$ ; weight  $\pm$  s.e.m. =  $32.2 \pm 2.9$  g) had transport parameters as follows (mean  $\pm$  s.e.m.): for the trachea  $Na^{+}$  =  $10.7 \pm 4.8$   $\mu A cm^{-2}$ ,  $Cl^{-}$  cAMP =  $11.4 \pm 4.3$   $\mu A cm^{-2}$ ,  $Cl^{-}$   $Ca^{2+}$  =  $12.1 \pm 4.7$   $\mu A cm^{-2}$ ; for the caecum  $Cl^{-}$  cAMP =  $35.6 \pm 7.0$   $\mu A cm^{-2}$ . Heterozygous (*cf/+*) mice transfected with pREP8-CFTR ( $n=2$ ; mean weight, 7.0 g) had the following transport parameters (mean  $\pm$  s.e.m.): for the trachea:  $Na^{+}$  =  $4.5$   $\mu A cm^{-2}$ ,  $Cl^{-}$  cAMP =  $6.6$   $\mu A cm^{-2}$ ,  $Cl^{-}$   $Ca^{2+}$  =  $8.2$   $\mu A cm^{-2}$ ; for the caecum  $Cl^{-}$  cAMP =  $104.6$   $\mu A cm^{-2}$ . Note that the forskolin-sensitive currents ( $Cl^{-}$  cAMP) in the trachea were smaller than those reported previously for wild-type mice<sup>17</sup>. This is undoubtedly a consequence of edge damage caused by using only 2.27 mm<sup>2</sup> areas of trachea. In the present study, necessitated by the small size of the *cf/cf* mice, compared with 4 mm<sup>2</sup> areas of trachea in previous studies.

**METHODS.** Transgenic mice were genotyped by PCR and/or Southern blot analysis as described<sup>17</sup>. Introduction of plasmid DNA into the mouse airways was as described in the legend to Fig. 2. Trachea and caeca were removed from the transfected animals killed by exposure to 100%  $CO_2$ . A single tracheal preparation (2.27 mm<sup>2</sup>) and a single caecal preparation (20 mm<sup>2</sup>) was prepared from each animal. The reduction in tracheal area, compared with a previous report<sup>17</sup> was due to the necessity of using animals as small as 5 g. The trachea were cleaned and cut longitudinally along the dorsal



surface and a piece placed under microscopic control in a specially constructed Ussing chamber designed to preserve the curvature of the tissue. Electrogenic ion transport was measured directly as SCC recorded by voltage clamping the tissue at zero potential, as described previously<sup>17</sup>.

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compared with the control (+/+) mice, confirming the genotypes of the mice and that the transfection procedure did not affect the gut. Thus, the transfection procedure used can restore CFTR-dependent, cAMP-stimulated chloride secretion by airway epithelia to normal levels.

In the airways of human CF patients there is an increase in amiloride-sensitive sodium absorption, as well as a decrease in chloride secretion, compared with controls<sup>18-20</sup>. It has been suggested that this is crucial to the development of the disease state, as application of amiloride by aerosol alleviates the decline in lung function in CF<sup>21,22</sup>. It is not yet clear how a loss of CFTR function leads to this increase in sodium absorption. In contrast to the human, sodium absorption was reduced in the airways of *cf/cf* mice (Fig. 4B). Transfection of the *cf/cf* mice with pREP8-CFTR, but not with the vector pREP8, significantly increased sodium absorption (seven- to eightfold;  $P < 0.05$ ), to essentially wild-type (+/+) levels (Fig. 4B). Thus, secondary alterations in sodium transport were also corrected to wild-type levels by the transfection protocol used. Finally,  $\text{Ca}^{2+}$ -induced chloride secretion reflects an alternative pathway for chloride secretion in the airways distinct from the CFTR pathway<sup>4,23</sup>.  $\text{Ca}^{2+}$ -stimulated chloride secretory currents were not defective in *cf/cf* trachea, compared with trachea of normal (+/+) mice, but were significantly increased following transfection with CFTR ( $P < 0.05$ ; Fig. 4B). This latter increase is probably a consequence of hyperpolarization through  $\text{Ca}^{2+}$ -sensitive  $\text{K}^+$  channels, which increases the electrochemical gradient for  $\text{Cl}^-$  exit through the introduced CFTR channels and the pre-existing second pathway<sup>24</sup>.

These data show that the ion transport defects in CF can be corrected *in vivo*. Liposomes, which in clinical trials have been shown to be non-toxic and non-immunogenic, may be safer than viral vectors which have the inherent risks of immunogenicity, replication and transmission. Our results illustrate the invaluable role of transgenic null *cf/cf* mice in assessing the efficiency of various gene therapy approaches. We have shown that functional expression of CFTR not only corrects the primary ion transport defect of the trachea (that is, the cAMP-stimulated chloride secretion), but also corrects secondary alterations in sodium absorption which are a consequence of loss of CFTR function. There seems to be no reason why this approach should not be transferable to humans for the treatment of the pulmonary features of CF. □

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## Germ-line transmission and expression of a human-derived yeast artificial chromosome

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INTRODUCTION of DNA fragments, hundreds of kilobases in size, into mouse embryonic stem (ES) cells would greatly advance the ability to manipulate the mouse genome. Mice generated from such modified cells would permit investigation of the function and expression of very large or crudely mapped genes. Large DNA molecules cloned into yeast artificial chromosomes (YACs) are stable and genetically manipulable within yeast<sup>1</sup>, suggesting yeast-cell fusion as an ideal method for transferring large DNA segments into mammalian cells. Introduction of YACs into different cell types by this technique has been reported<sup>2-4</sup>; however, the incorporation of yeast DNA along with the YAC has raised doubts as to whether ES cells, modified in this way, would be able to recolonize the mouse germ line<sup>5</sup>. Here we provide, to our knowledge, the first demonstration of germ-line transmission and expression of a large human DNA fragment, introduced into ES cells by fusion with yeast spheroplasts. Proper development was not impaired by the cointegration of a large portion of the yeast genome with the YAC.

Yeast spheroplasts, carrying yHPRT, a 670 kilobase (kb) YAC containing the human hypoxanthine phosphoribosyltransferase (HPRT) gene<sup>6</sup>, were fused with the HPRT-deficient ES cell line E14TG2a (ref. 9). Clones expressing the HPRT locus were selected in hypoxanthine/aminopterin/thymidine (HAT) medium (Fig. 1 legend) and expanded. The human HPRT gene was detected by hybridization in all ES cell clones analysed (not shown). The integration of additional human sequences was examined by comparing the *Alu* profile of 37 HAT-resistant (ESY) clones to that of yHPRT in yeast. Most, if not all, of the 30 *Alu* fragments characteristic of yHPRT were present and of similar relative intensity in over 90% of the ESY clones (Figs 1a, 3b). In clones with an incomplete *Alu* profile (such as ESY 8-5, Fig. 1a) only a few fragments were missing or altered in size. In most ESY clones, the *Alu* pattern appeared to be intact and without significant deletion, rearrangement or segmental amplification.

Integration of YAC vector sequences was investigated with vector arm-specific probes. A 4.5 kb *HindIII* fragment, detected by the right arm probe in yHPRT, was observed in 10 of 20 ESY clones (Fig. 1b). This vector arm was lost in eight ESY clones (for example ESY 3-1, 3-6, Fig. 1b) and rearranged in two (for example ESY 8-6, Fig. 1b). The left arm probe detected the 3 kb and 4.1 kb *HindIII* yHPRT fragments in 18 of 20 clones (Fig. 1c). In total, 8 of the 20 clones (such as ESY 5-2, 8-7, 7-3, Fig. 1a-c) contained complete *Alu* profiles and both intact YAC vector arms.

The structural integrity of yHPRT in ESY clones 5-2 and 8-7 was further evaluated by pulsed-field gel electrophoresis. In yeast carrying yHPRT, five *SfiI* fragments of the following rough sizes were defined by different probes: 315 kb (*Alu*, left arm),

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COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

**IN THE MATTER OF:** Australian  
Patent Application 696764  
(73941/94). In the name of:  
Human Genome Sciences Inc.

- and -

**IN THE MATTER OF:** Opposition  
thereto by Ludwig Institute for Cancer  
Research, under Section 59 of the  
Patents Act.

**Annexure GBC-15**

This is **Annexure GBC-15** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
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**Gary Baxter Cox**

WITNESS:

  
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Patent Attorney

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## REPORTS

phosphate-buffered saline (overnight). We added B19 empty capsids (100  $\mu$ l, 5  $\mu$ g/ml) to each well (in quadruplicate) and detected binding by a mouse anti-B19 monoclonal antibody (Chemicon) followed by  $^{125}$ I-labeled sheep anti-mouse antibody (Amersham). The radioactivity in each well was counted for 2 min with a  $\gamma$  counter. There was no specific binding with CDH or CTH (methanol alone, mean count of 241; CDH, mean count of 282; CTH, mean count of 244), and reduced binding to Forssman antigen (mean count of 2088) compared to globoside (mean count of 7458).

18. B19 capsids and mouse monoclonal antibody to globoside bound to the same band on Western blotting of red cell extract, perhaps to a putative "globoprotein" (Y. Tonegawa and S. Hakomori,

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## In Vivo Gene Therapy of Hemophilia B: Sustained Partial Correction in Factor IX-Deficient Dogs

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The liver represents a model organ for gene therapy. A method has been developed for hepatic gene transfer in vivo by the direct infusion of recombinant retroviral vectors into the portal vasculature, which results in the persistent expression of exogenous genes. To determine if these technologies are applicable for the treatment of hemophilia B patients, preclinical efficacy studies were done in a hemophilia B dog model. When the canine factor IX complementary DNA was transduced directly into the hepatocytes of affected dogs in vivo, the animals constitutively expressed low levels of canine factor IX for more than 5 months. Persistent expression of the clotting factor resulted in reductions of whole blood clotting and partial thromboplastin times of the treated animals. Thus, long-term treatment of hemophilia B patients may be feasible by direct hepatic gene therapy in vivo.

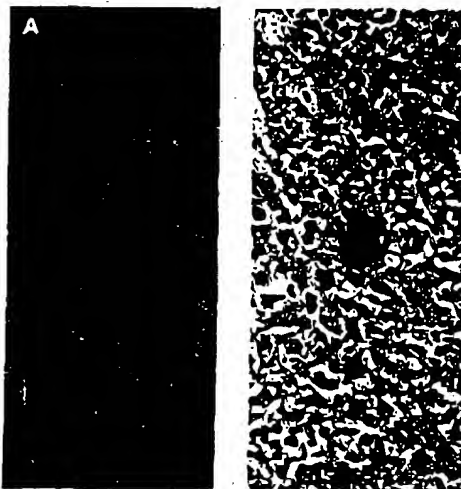
Hemophilia B is an X-linked blood coagulation disorder resulting from a deficiency of factor IX production in the liver. The disease affects about 1 in 30,000 males and can result in severe bleeding episodes that require infusion of blood products that contain factor IX (1). As a result of previous human protein replacement therapy, about half of hemophilia B patients are infected with human immunodeficiency virus or hepatitis viruses. A virus-free and non-thrombogenic factor IX product is now available, but because of high costs the current treatment protocols do not include prophylaxis and therapy is initiated after bleeding begins. A number of tissues are target organs for somatic gene therapy of hemophilia B, including fibroblasts, myoblasts, endothelial cells, keratinocytes, and hepatocytes (2-7).

laxis and therapy is initiated after bleeding begins. A number of tissues are target organs for somatic gene therapy of hemophilia B, including fibroblasts, myoblasts, endothelial cells, keratinocytes, and hepatocytes (2-7).

**Fig. 1.** Retroviral vector-mediated gene transfer of canine hepatocytes in vivo. A two-thirds partial hepatectomy was performed in two, 9-week-old normal dogs (3.5 kg) by resecting the left medial, left lateral, right medial, and caudate lobes. The right lateral lobe and its segmental blood supply and biliary drainage were preserved. The distal tip of a porta-cath catheter (Access Technology, Skokie, IL) was cannulated into a splenic vein. The injection port was placed subcutaneously under the right lateral abdominal wall. The LBGpgk vector was collected from confluent packaging cells cultured in Hg DMEM and 1% Hyclone for 12 hours. About 85 ml of filtered supernatants containing  $9 \times 10^7$  colony-forming units was mixed with Polybrene (20  $\mu$ g/ml) and infused over 45 to 90 min through the catheter 24, 48, and 72 hours after the hepatectomy. The animals tolerated the procedure well except for occasional vomiting and transient pallor during the beginning of the first infusion. When the dogs were killed, hepatocytes were isolated, cultured (70), and stained with x-Gal (8) (A) (original magnification,  $\times 200$ ), and (B) liver sections were stained with x-Gal and counterstained with neutral red (8) (original magnification,  $\times 400$ ).

Because the liver is the organ of factor IX synthesis, it represents a natural target for gene replacement therapy.

We have previously reported that hepatocytes can be transduced in vivo by infusion of recombinant retroviral vectors into the portal vasculature of mice after partial hepatectomy (8). To determine if the same can be achieved in larger animals (such as dogs), we infused an amphotropic retroviral vector (LBGpgk) that encodes the *Escherichia coli*  $\beta$ -galactosidase gene (8) directly into the portal vasculature of normal dogs three times 1 to 3 days after partial hepatectomy (Fig. 1). Two weeks later, hepatocytes were isolated and stained with 5-bromo-4-chloro-3-indolyl-6-D-galactopyranoside (x-Gal) (Fig. 1A). Liver sections from these animals were similarly analyzed (Fig. 1B). The proportion of stained (blue) cells in Fig. 1 represents the in vivo transduction frequency of hepatocytes and was about 1 and 0.3% in two animals. Additional tissues, including kidney and spleen, did not stain blue with x-Gal. These transduction efficiencies are similar to that previously observed in mice (8). Routine histologic analysis revealed no pathologic conditions in the liver.



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An amphotropic retroviral vector that encoded the canine factor IX complementary DNA (cDNA) (LX-cFIX) was constructed (9) and transduced into rat embryo 208F cells (8) to assess its ability for *in vitro* expression of factor IX. The media were changed daily, and canine factor IX was measured by enzyme-linked immunosorbent assay (ELISA) (8, 10) with a species-specific polyclonal antibody that was prepared as described (11). The transduced rodent cells produce 225 ng of canine factor IX antigen per  $10^6$  cells per day, whereas control cells produce no detectable factor IX (12). This recombinant retroviral vector was used for infusion into the portal vasculature of four hemophilia B dogs (Table 1) from the Chapel Hill inbred strain (13). The molecular defect in these dogs is a missense mutation in the catalytic domain of factor IX that results in a complete lack of antigen in the plasma (14). A partial hepatectomy was performed in these experimental ani-

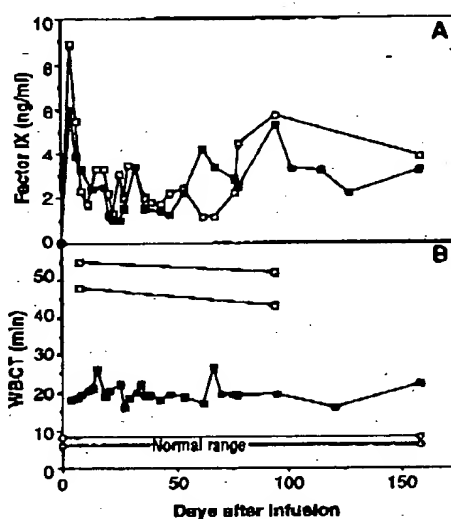
mals, followed by the infusion of the LX-cFIX retrovirus 24, 48, and 72 hours after partial hepatectomy (Table 1). Factor IX concentrations in their plasma were measured by biologic and immunoassays (Figs. 2A and 3A). The hemostatic parameters were monitored by changes in the whole blood clotting time (WBCT) (Figs. 2B and 3B) and partial thromboplastin time (PTT) (Table 1). These tests are indexes of the intrinsic pathway of clotting in which factor IX is the key component.

In dog 1, the plasma factor IX increased from undetectable amounts to a range of 2 to 6 ng/ml; these levels have been maintained constitutively for over 5 months (Fig. 2A), and there is close agreement between the biologic and immunoassay results. Most importantly, this dog had a WBCT of 15 to 20 min during the 5-month period after treatment, whereas the WBCT for untreated factor IX-deficient littermates ranged from 45 to 55 min

(Fig. 2B). A long WBCT is characteristic of a dog colony severely deficient in factor IX; normal dogs, on the other hand, have a WBCT of 6 to 8 min (Fig. 2B). *In vivo* addition of normal dog plasma to whole blood from hemophilia B dogs to a final concentration of 3 ng/ml reduced the WBCT to 20 min. This is in agreement with the WBCT and factor IX concentrations obtained in dog 1 after treatment (Fig. 2). The PTT for dog 1 was also shortened from 322 before treatment to 174 seconds after treatment (Table 1).

Plasma from dog 2 showed factor IX concentrations similar to that of dog 1 (Figs. 2A and 3A), whereas dog 3 had slightly greater concentrations, ranging from 3 to 10 ng/ml (Fig. 3A). Both dogs 2 and 3 had similar reductions in their WBCT, from pretreatment values of 44 to 47 min to 18 to 26 min after treatment (Fig.

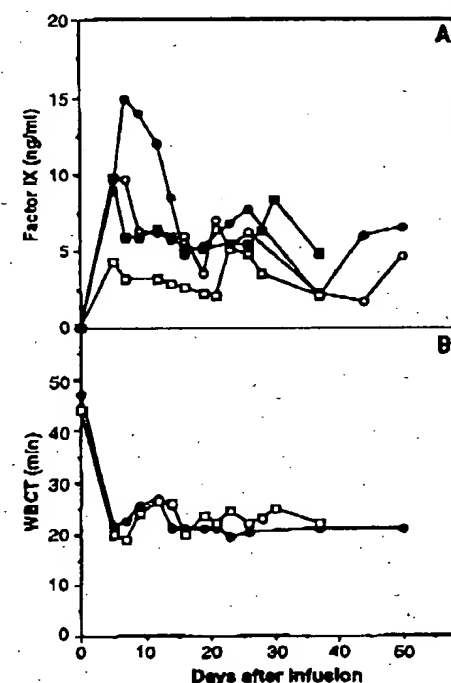
**Fig. 2.** Plasma factor IX concentrations and WBCTs in hemophilia B dog 1 after hepatic transduction with the LTR-cFIX retroviral vector. The plasma factor IX concentrations were determined immunologically by ELISA and by bioassay. The bioassays were performed by a modified one-stage method (16) in which a kaolin-activated canine hemophilia B plasma was used as a substrate, a limiting dilution of a normal canine plasma pool was used as reference plasma (11.5  $\mu$ g/ml), and test plasmas were diluted in a 1:10 ratio. The value of 11.5  $\mu$ g/ml was estimated from the ratio of the concentration of normal human factor IX activity (5.0  $\mu$ g/ml) to the concentration of normal canine factor IX activity. Hemostatic testing consisted of a determination of the WBCT (with the two-tube method at 28°C with the use of silicone-coated tubes filled every 30 s until clotted). (A) Values for the antigen concentrations of factor IX are shown in solid symbols, and the values from the bioassay are shown in open symbols. (B) The WBCTs in dog 1 (solid squares), two affected hemophilia B littermates (open squares), and the range in normal dogs (open circles). The days after infusion represent the number of days after the first retroviral infusion; day 0 represents measurements from samples obtained before any procedural manipulations.



**Table 1.** Hemophilia B dogs used in a gene therapy protocol (23). The weight and age were recorded on the day the study was started. The methods for partial hepatectomy were essentially the same as outlined in Fig. 1. Blood samples were obtained from the animals before surgery, and hemostatic coverage was maintained with multiple infusions of normal fresh frozen plasma given immediately before and for 24 hours after the operation (57.4 to 72.7 units of factor IX per kilogram of body weight). Circulating factor IX from plasma infusions in untreated animals is cleared within 8 to 10 days (22). The PTTs (24) were obtained from two to four samples before the start of the experiments (Before) and then were analyzed 6 to 10 times for each animal on different days starting at least 11 days after the viral infusion (After). The times shown indicate nonactivated PTTs, which for normal dogs are 42 to 47 s. The standard deviations are in parentheses.

Dog*	Weight (kg)	Age (weeks)	Infusion volume (ml)	PTT (s)	
				Before	After
1	5.3	11	510	322 (15)	174 (6.5)
2	7.3	14	720	282 (0.8)	190 (7.5)
3	6.7	14	720	195 (13)	154 (8.6)

\*One animal died as a result of a surgical complication and was not included.



**Fig. 3.** Plasma factor IX concentrations and WBCTs in hemophilia B dogs 2 and 3 after hepatic transduction with the LTR-cFIX retroviral vector. The hemostatic characterization were as described (Fig. 2). (A) The values for the antigen concentrations are in solid symbols. Dog 2, squares; dog 3, circles. Values from the bioassay are in open symbols. (B) Whole blood clotting times. Dog 2, open squares; dog 3, solid circles. The study on dog 2 terminated on day 42 as multiple transfusions were given to hemostasis. Dog 3 developed a minor bleeding episode that required one infusion of normal dog plasma on day 26, which accounts for the hiatus in data for days 27 to 36. Previous studies (22) have shown that by 10 days after infusion exogenous factor IX is undetectable by bioassay.



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3B). The PTTs in dogs 2 and 3 were also decreased significantly (Table 1). To further establish the biological activity of plasma factor IX, we treated samples from dogs 1 and 3 with barium sulfate to remove  $\gamma$ -carboxylated proteins, including factor IX (15). The barium sulfate-treated plasmas of treated dogs had a prolonged PTT that was similar to the pretreatment PTT values.

Our study demonstrates the feasibility of in vivo retroviral-mediated gene transfer into the liver of a large animal, which results in phenotypic improvement of a deficiency syndrome. The factor IX antigen amounts achieved after gene transfer were only about 0.1% of the endogenous concentration of factor IX in normal animals, which demonstrates that the constitutive expression of a relatively small quantity of the factor IX protein is sufficient to cause a reduction in the WBCT and a shortening of the PTT. Our data indicate that the WBCT is extremely sensitive to changes in factor IX concentration in the hemophilia B dogs. In moderate and mild human hemophilia patients with shortened WBCT and factor levels in the 3 to 8% range, there is still a risk of hemorrhage, although both the frequency and the severity of episodes are considerably less than those of severely affected individuals (16). For future human applications, however, increased circulating factor IX levels must first be achieved. This may be accomplished by developing methods that lead to greater efficiencies of hepatocyte transduction in vivo and by creating expression vectors with stronger promoters. These reservations notwithstanding, our results illustrate the efficacy of in vivo gene therapy of hemophilia B and other metabolic disorders secondary to hepatic deficiencies.

*Note added in proof:* Plasma factor IX concentrations and WBCTs remained at the

same values at 9 months after treatment for dog 1 and 6 months after treatment for dog 3.

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21. Viral supernatants were obtained from clonal packaging cell lines and diluted by 10-fold serial dilutions and infected 208F cells (20). Factor IX concentrations in the media were detected by ELISA, and 3 days after infection the cells were isolated and DNA extracted for PCR amplification with primers specific for the canine factor IX cDNA. We compared the PCR signal of the LX-cFIX-infected cells with that of a retroviral vector that contains the cFIX cDNA and a selectable marker that has been titrated by standard methods (20) and is known to have a titer of  $2 \times 10^7$  colony-forming units per milliliter. The PCR signal for the LX-cFIX vector is not detectable one dilution before the vector with a known titer. Thus, the titer of the LX-cFIX virus is estimated to be  $2 \times 10^6$  infectious particles per milliliter.
22. K. M. Brinkhous, C. N. Landen, M. S. Reed, unpublished data.
23. All experimental procedures were in accordance with institutional guidelines at the Baylor College of Medicine and the University of North Carolina. Pre-anesthetic agents included atropine sulfate (0.24 mg per kilogram of body weight) and sodium thiamylal (0.1 to 0.2 g/kg). Maintenance anesthesia was 2% halothane. The postoperative analgesic was butorphanol (0.5 mg) administered intravenously as needed every 4 to 6 hours during the first 24 hours. The animals were treated prophylactically with antibiotics (cefadroxil, 22 mg/kg) for several days before and after the operation. Before retroviral infusion, the animals were given diphenhydramine (10 mg) intramuscularly, and some animals were sedated with acepromazine (5 mg) intravenously.
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25. We thank L. Brandon and A. Major (Houston, TX) and S. Olgesbee, A. McCrory, R. Raymer, and P. McElveen (Chapel Hill, NC) for their expert technical assistance. Supported in part by NIH grants DK 44080, HL 40162 (Baylor College of Medicine), and HL-01848-46 and HL 26309-12 (University of North Carolina, Chapel Hill). M.A.K. is a recipient of an individual National Research Service Awards fellowship award GM 13894, and S.L.C.W. is an investigator of the Howard Hughes Medical Institute.

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## Science

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### In Vivo Gene Therapy of Hemophilia B: Sustained Partial Correction in Factor IX-Deficient Dogs [REPORTS]

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#### Outline

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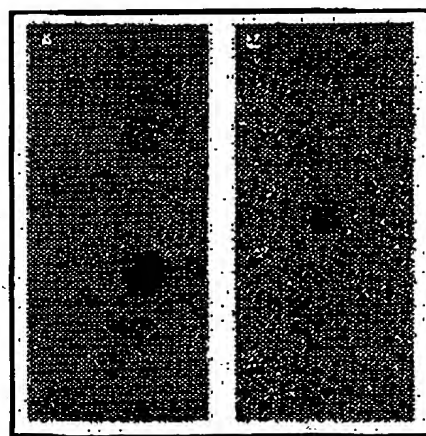
#### Abstract:

The liver represents a model organ for gene therapy. A method has been developed for hepatic gene transfer in vivo by the direct infusion of recombinant retroviral vectors into the portal vasculature, which results in the persistent expression of exogenous genes. To determine if these technologies are applicable for the treatment of hemophilia B patients, preclinical efficacy studies were done in a hemophilia B dog model. When the canine factor IX complementary DNA was transduced directly into the hepatocytes of affected dogs in vivo, the animals constitutively expressed low levels of canine factor IX for more than 5 months. Persistent expression of the clotting factor resulted in reductions of whole blood clotting and partial thromboplastin times of the treated animals. Thus, long-term treatment of hemophilia B patients may be feasible by direct hepatic gene therapy in vivo.

Hemophilia B is an X-linked blood coagulation disorder resulting from a deficiency of

factor IX production in the liver. The disease affects about 1 in 30,000 males and can result in severe bleeding episodes that require infusion of blood products that contain factor IX [1]. As a result of previous human protein replacement therapy, about half of hemophilia B patients are infected with human immunodeficiency virus or hepatitis viruses. A virus-free and nonthrombogenic factor IX product is now available, but because of high costs the current treatment protocols do not include prophylaxis and therapy is initiated after bleeding begins. A number of tissues are target organs for somatic-gene therapy of hemophilia B, including fibroblasts, myoblasts, endothelial cells, keratinocytes, and hepatocytes [2-7]. Because the liver is the organ of factor IX synthesis, it represents a natural target for gene replacement therapy.

We have previously reported that hepatocytes can be transduced in vivo by infusion of recombinant retroviral vectors into the portal vasculature of mice after partial hepatectomy [8]. To determine if the same can be achieved in larger animals (such as dogs), we infused an amphotropic retroviral vector (LBGpgk) that encodes the *Escherichia coli* beta-galactosidase gene [8] directly into the portal vasculature of normal dogs three times 1 to 3 days after partial hepatectomy *Figure 1*. Two weeks later, hepatocytes were isolated and stained with 5-bromo-4-chloro-3-indoyl-6-D-galactopyranoside (x-Gal) *Figure 1A*. Liver sections from these animals were similarly analyzed *Figure 1B*. The proportion of stained (blue) cells in *Figure 1* represents the in vivo transduction frequency of hepatocytes and was about 1 and 0.3% in two animals. Additional tissues, including kidney and spleen, did not stain blue with x-Gal. These transduction efficiencies are similar to that previously observed in mice [8]. Routine histologic analysis revealed no pathologic conditions in the liver.



**Figure 1.** Retroviral vector-mediated gene transfer of canine hepatocytes in vivo. A two-thirds partial hepatectomy was performed in two, 9-week-old normal dogs (3.5 kg) by resecting the left medial, left lateral, right medial, and caudate lobes. The right lateral lobe and its segmental blood supply and biliary drainage were preserved. The distal tip of a porta-cath catheter (Access Technology, Skokie, IL) was cannulated into a splenic vein. The injection port was placed subcutaneously under the right lateral abdominal wall. The LBGpgk vector was collected from confluent packaging cells cultured in Hg DMEM and 1% Hyclone for 12 hours. About 85 ml of filtered supernatants containing  $9 \times 10^7$  colony-forming units was mixed with Polybrene (20  $\mu$ g/ml) and infused over 45 to 90 min through the catheter 24, 48, and 72 hours after the hepatectomy. The animals tolerated the procedure well except for occasional vomiting and transient pallor during the beginning of the first infusion. When the dogs were killed, hepatocytes were isolated, cultured [10], and stained with x-Gal [8] (A) (original magnification, times 200), and (B) liver sections were stained with x-Gal and counterstained with neutral red [8] (original magnification, times 400).

An amphotropic retroviral vector that encoded the canine factor IX complementary DNA (cDNA) (LX-cFIX) was constructed [9] and transduced into rat embryo 208F cells [8] to assess its ability for in vitro expression of factor IX. The media were changed daily, and canine factor IX was measured by enzyme-linked immunosorbent assay (ELISA) [8,10] with a species-specific polyclonal antibody that was prepared as described [11]. The transduced rodent cells produce 225 ng of canine factor IX antigen per  $10^6$  cells per day, whereas control cells produce no detectable factor IX [12]. This recombinant retroviral vector was used for infusion into the portal vasculature of four hemophilia B dogs *Table 1* from the Chapel Hill inbred strain [13]. The molecular defect in these dogs is a missense mutation in the catalytic domain of factor IX that results in a complete lack of antigen in the plasma [14]. A partial hepatectomy was performed in these experimental animals, followed by the infusion of the LX-cFIX retrovirus 24, 48, and 72 hours after partial hepatectomy *Table 1*. Factor IX concentrations in their plasma were measured by biologic and immunoassays *Figure s. 2A and 3A*). The hemostatic parameters were monitored by changes in the whole blood clotting time (WBCT) (*Figs. 2B and 3B*) and partial thromboplastin time (PTT) *Table 1*. These tests are indexes of the

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intrinsic pathway of clotting in which factor IX is the key component.

Weight (kg)	Age (days)	Sex	Factor IX concentration (mu/g/ml)	PTT (s)
12.5	120	M	11.5	42
12.5	120	F	11.5	42
12.5	120	M	11.5	42
12.5	120	F	11.5	42
12.5	120	M	11.5	42
12.5	120	F	11.5	42
12.5	120	M	11.5	42
12.5	120	F	11.5	42
12.5	120	M	11.5	42
12.5	120	F	11.5	42

Table 1. Hemophilia B dogs used in a gene therapy protocol [23]. The weight and age were recorded on the day the study was started. The methods for partial hepatectomy were essentially the same as outlined in Figure 1. Blood samples were obtained from

the animals before surgery, and hemostatic coverage was maintained with multiple infusions of normal fresh frozen plasma given immediately before and for 24 hours after the operation (57.4 to 72.7 units of factor IX per kilogram of body weight). Circulating factor IX from plasma infusions in untreated animals is cleared within 8 to 10 days [22]. The PTTs [24] were obtained from two to four samples before the start of the experiments (Before) and then were analyzed 6 to 10 times for each animal on different days starting at least 11 days after the viral infusion (After). The times shown indicate nonactivated PTTs, which for normal dogs are 42 to 47 s. The standard deviations are in parentheses.

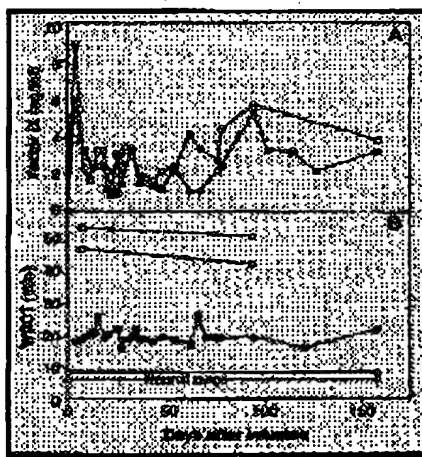


Figure 2. Plasma factor IX concentrations and WBCTs in hemophilia B dog 1 after hepatic transduction with the LTR-cFIX retroviral vector. The plasma factor IX concentrations were determined immunologically by ELISA and by bioassay. The bioassays were performed by a modified one-stage method [16] in which a kaolin-activated canine hemophilia B plasma was used as a substrate, a limiting dilution of a normal canine plasma pool was used as reference plasma (11.5 mu/g/ml), and test plasmas were diluted in a 1:10 ratio. The value of 11.5 mu/g/ml was estimated from the ratio of the concentration of normal human factor IX activity (5.0 mu/g/ml) to the concentration of normal canine factor IX activity. Hemostatic testing consisted of a determination of the WBCT (with the two-tube method at 28 degrees C with the use of silicone-coated tubes tilted every 30 s until clotted). (A) Values for the antigen concentrations of factor IX are shown in solid symbols, and the values from the bioassay are shown in open symbols. (B) The WBCTs in dog 1 (solid squares), two affected hemophilia B littermates (open squares), and the range in normal dogs (open circles). The days after infusion represent the number of days after the first retroviral infusion; day 0 represents measurements from samples obtained before any procedural manipulations.

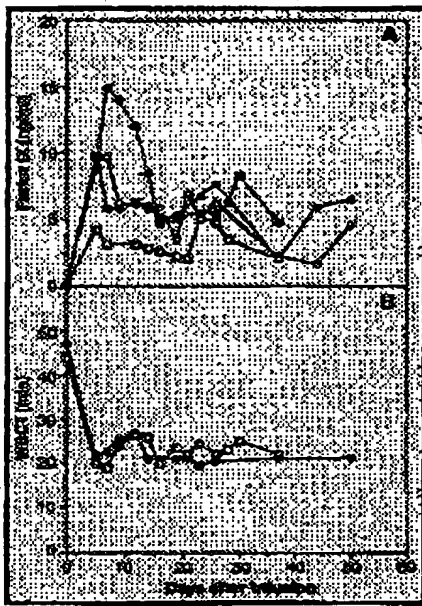


Figure 3. Plasma factor IX concentrations and WBCTs in hemophilia B dogs 2 and 3 after hepatic transduction with the LTR-cFIX retroviral vector. The hemostatic characterizations were as described Figure 2. (A) The values for the antigen concentrations are in solid symbols. Dog 2, squares; dog 3, circles. Values from the bioassay are in open symbols. (B) Whole blood clotting times. Dog 2, open squares; dog 3, solid circles. The study on dog 2 terminated on day 42 as multiple transfusions were given for hemorrhage. Dog 3 developed a minor bleeding episode that required one infusion of normal dog plasma on day 26, which accounts for the hiatus in data for days 27 to 36. Previous studies [22] have shown that by 10 days after infusion exogenous factor IX is undetectable by bioassay.

In dog 1, the plasma factor IX increased from undetectable amounts to a range of 2 to 6 ng/ml; these levels have been maintained constitutively for over 5 months (Figure 2A) and there is close agreement between the biologic and immunoassay results. Most importantly, this dog had a WBCT of 15 to 20 min during the 5-month period after treatment, whereas the WBCT for untreated factor IX-deficient littermates ranged from 45 to 55 min (Figure 2B). A long WBCT is characteristic of a dog colony severely deficient in factor IX; normal dogs, on the other hand, have a WBCT of 6 to 8 min (Figure 2C). *In vitro* addition of normal dog plasma to whole blood from hemophilia B dogs to a final concentration of 3 ng/ml reduced the WBCT to

20 min. This is in agreement with the WBCT and factor IX concentrations obtained in dog 1 after treatment [Figure 2](#). The PTT for dog 1 was also shortened from 322 before treatment to 174 seconds after treatment [Table 1](#).

Plasma from dog 2 showed factor IX concentrations similar to that of dog 1 (Figs. 2A and 3A), whereas dog 3 had slightly greater concentrations, ranging from 3 to 10 ng/ml [Figure 3A](#). Both dogs 2 and 3 had similar reductions in their WBCT, from pretreatment values of 44 to 47 min to 18 to 26 min after treatment [Figure 3B](#). The PTTs in dogs 2 and 3 were also decreased significantly [Table 1](#). To further establish the biological activity of plasma factor IX, we treated samples from dogs 1 and 3 with barium sulfate to remove gamma-carboxylated proteins, including factor IX [\[15\]](#). The barium sulfate-treated plasmas of treated dogs had a prolonged PTT that was similar to the pretreatment PTT values.

Our study demonstrates the feasibility of in vivo retroviral-mediated gene transfer into the liver of a large animal, which results in phenotypic improvement of a deficiency syndrome. The factor IX antigen amounts achieved after gene transfer were only about 0.1% of the endogenous concentration of factor IX in normal animals, which demonstrates that the constitutive expression of a relatively small quantity of the factor IX protein is sufficient to cause a reduction in the WBCT and a shortening of the PTT. Our data indicate that the WBCT is extremely sensitive to changes in factor IX concentration in the hemophilia B dogs. In moderate and mild human hemophilia patients with shortened WBCT and factor levels in the 3 to 8% range, there is still a risk of hemorrhage, although both the frequency and the severity of episodes are considerably less than those of severely affected individuals [\[16\]](#). For future human applications, however, increased circulating factor IX levels must first be achieved. This may be accomplished by developing methods that lead to greater efficiencies of hepatocyte transduction in vivo and by creating expression vectors with stronger promoters. These reservations notwithstanding, our results illustrate the efficacy of in vivo gene therapy of hemophilia B and other metabolic disorders secondary to hepatic deficiencies.

Note added in proof: Plasma factor IX concentrations and WBCTs remained at the same values at 9 months after treatment for dog 1 and 6 months after treatment for dog 3.

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9. In the construction of the LX-cFIX retroviral vector, the 1.4-kb cFIX cDNA was cloned by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of canine liver polyadenylate-containing RNA. The 5 prime primer

was synthesized to contain the 5 prime-GCCACC-3 prime Kozak sequence [M. Kozak, Nucleic Acids Res. 15, 8125 (1987)]. The sequence of the cDNA was determined and found to be identical to the published sequence \*RF 14\*. The cDNA was cloned into a modified LNCX retroviral vector \*RF 17\*, with its neo gene and cytomegalovirus promoter removed by Bcl I-Bam HI digestion and religation. The cloned DNA was transfected into GP plus E-86 ecotropic packaging cells \*RF 18\*, and supernatants were used to infect amphotropic GP plus AM12 cells \*RF 19\*. The single clone that gave the greatest amount of cFIX production in transduced 208F cells was titrated \*RF 20\* by limiting dilution PCR \*RF 21\* and found to have a concentration of 2 times 10 sup 6 colony-forming units per milliliter. [Context Link]

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12. Viral supernatants contained Polybrene (8 mu g/ml) (Sigma) at a multiplicity of infection of 10 \*RF 8\*. Control cells were infected with an equal amount of LBGpgk virus. The supernatants from transduced cells were collected at 24-hour intervals and analyzed for cFIX. The LBGpgk-infected cells were stained with x-Gal, and approximately 30 to 40% of these cells stained blue. [Context Link]

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19. \_\_\_\_\_, Virology 167, 400 (1988).

20. C. M. Lynch and A. D. Miller, J. Virol. 65, 3887 (1991).

21. Viral supernatants were obtained from clonal packaging cell lines and diluted by 10-fold serial dilutions and infected 208F cells \*RF 20\*. Factor IX concentrations in the media were detected by ELISA, and 3 days after infection the cells were isolated and DNA extracted for PCR amplification with primers specific for the canine factor IX cDNA. We compared the PCR signal of the LX-cFIX-infected cells with that of a retroviral vector that contains the cFIX cDNA and a selectable marker that has been titrated by standard methods \*RF 20\* and is known to have a titer of 2 times 10 sup 7 colony-forming units per milliliter. The PCR signal for the LX-cFIX vector is not detectable one dilution before the vector with a known titer. Thus, the titer of the LX-cFIX virus is estimated to be 2 times 10 sup 6 infectious particles per milliliter.

22. K. M. Brinkhous, C. N. Landen, M. S. Read, unpublished data. [Context Link]

23. All experimental procedures were in accordance with institutional guidelines at the Baylor College of Medicine and the University of North Carolina. Preanesthetic agents included atropine sulfate (0.24 mg per kilogram of body weight) and sodium thiamylal (0.1 to 0.2 g/kg). Maintenance anesthesia was 2% halothane. The postoperative analgesic was butorphanol (0.5 mg) administered intravenously as needed every 4 to 6 hours during the first 24 hours. The animals were treated prophylactically with antibiotics (cefadroxil, 22 mg/kg) for several days before and after the operation. Before retroviral infusion, the animals were given diphenhydramine (10 mg) intramuscularly, and some animals were sedated with acepromazine (5 mg) intravenously. [Context Link]

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25. We thank L. Brandon and A. Major (Houston, TX) and S. Olgesbee, A. McCrory, R. Raymer, and P. Mcclveen (Chapel Hill, NC) for their expert technical assistance. Supported in part by NIH grants DK 44080, HL 40162 (Baylor College of Medicine), and HL-01648-46 and HL 26309-12 (University of North Carolina, Chapel Hill). M.A.K. is a recipient of an individual National Research Service Awards fellowship award GM 13894, and S.L.C.W. is an investigator of the Howard Hughes Medical Institute.

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COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

IN THE MATTER OF: Australian

Patent Application 696764

(73941/94). In the name of:

Human Genome Sciences Inc.

- and -

IN THE MATTER OF: Opposition

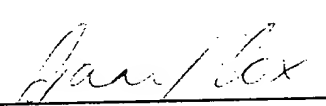
thereto by Ludwig Institute for Cancer

Research, under Section 59 of the

Patents Act.

Annexure GBC-16

This is **Annexure GBC-16** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
\_\_\_\_\_  
**Gary Baxter Cox**

WITNESS:

  
\_\_\_\_\_  
Patent Attorney

PEYTEE KHOW



## Hepatic Gene Therapy: Efficient Retroviral-Mediated Gene Transfer into Rat Hepatocytes In Vivo

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**Abstract**—The rat is an excellent model for gene therapy because there are many rat models for human diseases. We have developed a simple and efficient method to deliver genes to the rat liver using recombinant retroviral vectors. A 70% partial hepatectomy followed by retroviral infusion into the portal vein results in 10–15% hepatocyte transduction in vivo. This is 10 times more efficient than in the mouse due partially to the observation that the rat livers have much more synchronous hepatocyte replication after partial hepatectomy. Using a recombinant retroviral vector containing the human  $\alpha_1$ -antitrypsin cDNA, persistent expression of the human protein in recipient rat plasma was observed for at least six months and at a level that is 10 times greater than the mouse. Thus, rats can serve as an excellent model for gene therapy of metabolic disorders secondary to hepatic deficiencies.

### INTRODUCTION

The rat is an extensively investigated animal, and there are many excellent rat models of human genetic and epigenetic diseases (1). In order to investigate the feasibility of treating some of these disease models using the techniques of somatic cell gene transfer, we wanted to develop simple and efficient methods for gene therapy in the rat. An excellent target organ for gene therapy is the liver because it is a large, metabolically active organ, and many genetic diseases result from mutations in genes expressed in the liver (2). Hepatocytes also have a slow turnover rate (3), and thus genetically modified cells will persist long-term.

Presently, the best vector for stable introduction of genes into somatic cells in animals is the recombinant, replication-

defective retrovirus. The retrovirus is attractive because it integrates into the host genome and is thus permanent. The problem with using retroviruses to transduce hepatocytes is that retroviruses require host cell division for efficient integration into the genome (5). However, in a normal, healthy liver, very few hepatocytes are replicating (3). To stimulate hepatocyte replication, several laboratories have reported performing a 70% partial hepatectomy on rats. When the remaining hepatocytes divide to regenerate the liver, they are susceptible to retroviral transduction. The retrovirus is delivered by vascular isolation of the liver, followed by perfusion of the liver with retroviral supernatant (6, 7). Although this is an efficient method for gene delivery (5–20% of the hepatocytes were transduced), it is complicated by the need for extensive and elaborate surgery.

Recently, Kay et al. (8) performed a 70% partial hepatectomy in mice followed by infusion of retrovirus into the portal vein without vascular isolation. Using a retrovirus encoding the  $\beta$ -galactosidase gene, this technique resulted in 1–2% hepatocyte transduction as determined by X-gal staining. They also introduced the gene for the secreted reporter protein human  $\alpha_1$ -antitrypsin (hAAT) and detected constitutive levels of hAAT in mouse serum for more than 200 days. This method, applied in the rat, resulted in 10–15% hepatocyte transduction and constitutive expression of the human protein at a level 10-fold greater than in the mouse.

## MATERIALS AND METHODS

**Animals and Partial Hepatectomy.** Male Lewis rats, 3–4 weeks old were purchased from Harlan, Sprague Dawley Inc. The animals were housed in a vivarium with a 12-h light–dark cycle with water and food (standard laboratory chow) provided *ad libitum*. The partial hepatectomy was performed under a general combination anesthetic: ketamine (42.8 mg/ml), xylazine (8.6 mg/ml), and acepromazine (1.4 mg/ml), administered at 0.5–0.7 ml/kg. The 70% partial hepatectomy involved removal of the median and left lateral lobes and was performed according to Higgins and Anderson (9). The skin was then closed with autoclip 9-mm wound clips.

**Retrovirus Harvest and Preparation.** The virus-producing cells were cultured at 37°C with 5.0% CO<sub>2</sub> in 150-mm tissue culture plates with 25 ml of media (high glucose D-modified Eagle's media supplemented with 10% Hyclone bovine calf serum and 1 mM glutamine, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin). When the cells were 70–80% confluent, the medium was replaced with 15 ml of fresh medium; 18 h later, the medium was harvested, filtered through a 0.45- $\mu$ m syringe filter, and polybrene was

added to 8.0  $\mu$ g/ml. The retroviral medium was infused into animals within 1 h of collection.

**Infusion of Retrovirus into Remaining Lobes of Liver.** At indicated times after the 70% partial hepatectomy, rats were anesthetized as above and opened through the same incision used for the partial hepatectomy. The portal vein was cannulated with a 24-G catheter connected to a 10-cc syringe by a 30-in. extension set. Over the course of 20–30 min, 3.0 ml of retroviral supernatant was infused using a Sage Instruments syringe pump (model 355). After infusion, the catheter was removed and pressure was applied for 10–30 min to control bleeding. The abdominal muscle was sutured with Chromic Gut and the skin closed with autoclips.

**Isolation and X-Gal Staining of Hepatocytes.** The technique for hepatocyte isolation is adapted from Berry and Friend (10). Briefly, 10 days after retroviral infusion, the rat was anesthetized, the portal vein was cannulated with a 20-G catheter, and the inferior vena cava cut. The liver was then perfused with 150 ml of Earle's balanced salt solution without calcium or magnesium (EBS<sup>-</sup>) plus 0.5 mM EGTA, 50 ml of EBS<sup>-</sup>, and finally 150 ml of Earle's buffered solution with calcium, with 0.3 mg/ml Boehringer collagenase and 0.05 mg/ml Sigma soybean trypsin inhibitor. All the above solutions were warmed to 37°C and infused at 20 ml/min. Hepatocyte culture conditions and media were described previously (11). The hepatocytes were cultured for 16–20 h before X-gal staining, performed as described previously. Histological X-gal staining of frozen liver sections was performed seven days after retroviral infusion and was described previously (11).

**Generation of LX/hAAT Retrovirus.** LX/hAAT was constructed by removing the *neo* gene and CMV promoter from LNCX by a BclI (blunted), HindIII restriction enzyme digest, and inserting the hAAT coding region

as a *Sma*I, *Hind*III fragment. The LX/hAAT construct was then electroporated into GPAM-12 packaging cells, and individual virus producing colonies were selected and screened for retroviral production (retroviral supernatant from individual colonies were used to transduce rat embryo fibroblast cell line 208F and hAAT production from the fibroblasts was assayed). The apparent titer of LX/hAAT is at least  $2 \times 10^6$  PFU/ml as determined by comparing the amounts of viral RNA in the supernatant from LX/hAAT producing cells to a retrovirus of known titer (data not shown).

**Detection of hAAT in Rat Serum.** Rat serum was isolated and the concentration of human  $\alpha_1$ -antitrypsin was determined by an ELISA as described in Kay et al. (8).

## RESULTS

**Recombinant Retroviral Vectors.** The amphotropic retrovirus LX/ $\beta$ -Geo (Fig. 1A) has been described previously (8). Briefly,  $\beta$ -Geo is a fusion of the *E. coli*  $\beta$ -galactosidase gene and the neomycin phosphotransferase gene. The fusion protein retains both enzymatic activities. The  $\beta$ -Geo gene is under the transcriptional control of the Moloney murine leukemia virus (MMLV) long terminal repeat (LTR) described by Miller and Rosman (12). The retrovirus is produced from the amphotropic retroviral packaging cell line GPAM-12 and has a titer of  $1 \times 10^6$  PFU/ml. The LX/hAAT retrovirus (Fig. 1B) encodes the human  $\alpha_1$ -antitrypsin gene (8) under the transcriptional control of the MMLV-LTR (12).

**In Vivo Retroviral Transduction of Rat Hepatocytes.** The MMLV vectors require a cell to be dividing in order to integrate into the host genome. To stimulate hepatocyte replication, a 70% partial hepatectomy can be performed. Since young rats (3–4 weeks old) have a greater rate of DNA synthesis than older rats after partial hepatectomy (13), their hepatocytes will be more suscep-

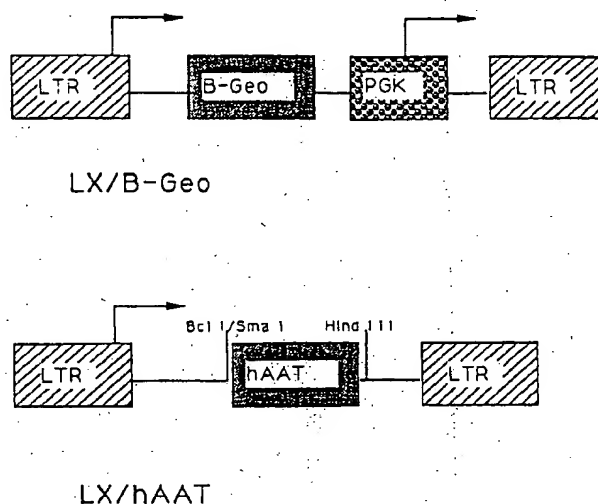


Fig. 1. Diagrams of the retroviruses used in this study. (A) LX/ $\beta$ -Geo,  $\beta$ -Geo is a fusion gene of the neomycin phosphotransferase gene and the  $\beta$ -galactosidase gene.  $\beta$ -Geo is under the transcriptional control of the Moloney murine leukemia virus (MMLV) long terminal repeat (LTR). The retrovirus also encodes for the phosphoglycerol kinase promoter (PGK). (B) LX/hAAT encodes the human  $\alpha_1$ -antitrypsin gene under the transcriptional control of MMLV LTR. The arrows indicate transcriptional start sites.

tible to retroviral transduction. A 70% partial hepatectomy was thus performed on 3 to 4-week-old male Lewis rats and 24 h later, 3 ml of the  $\beta$ -Geo retrovirus was infused into the portal vein over the course of 30 min. Seven days later, the liver was isolated and the frozen section was stained for X-gal. As can be seen in Fig. 2A, there are many cells that have stained in the liver infused with the  $\beta$ -Geo retrovirus, while no blue cells are visible in liver after only a partial hepatectomy and mock infusion (Fig. 2B). It is known that the amphotropic retrovirus can transduce vascular endothelial cells in vivo (4). However, of the sections we inspected, transduction was limited to cells with hepatocyte morphology (Fig. 2A). The blue cells are relatively evenly dispersed in the liver parenchyma, although some appeared in groups and rows, perhaps indicating limited division of hepatocytes following transduction.

**Time Course for Optimal Retroviral Transduction of Rat Hepatocytes In Vivo.** To deter-

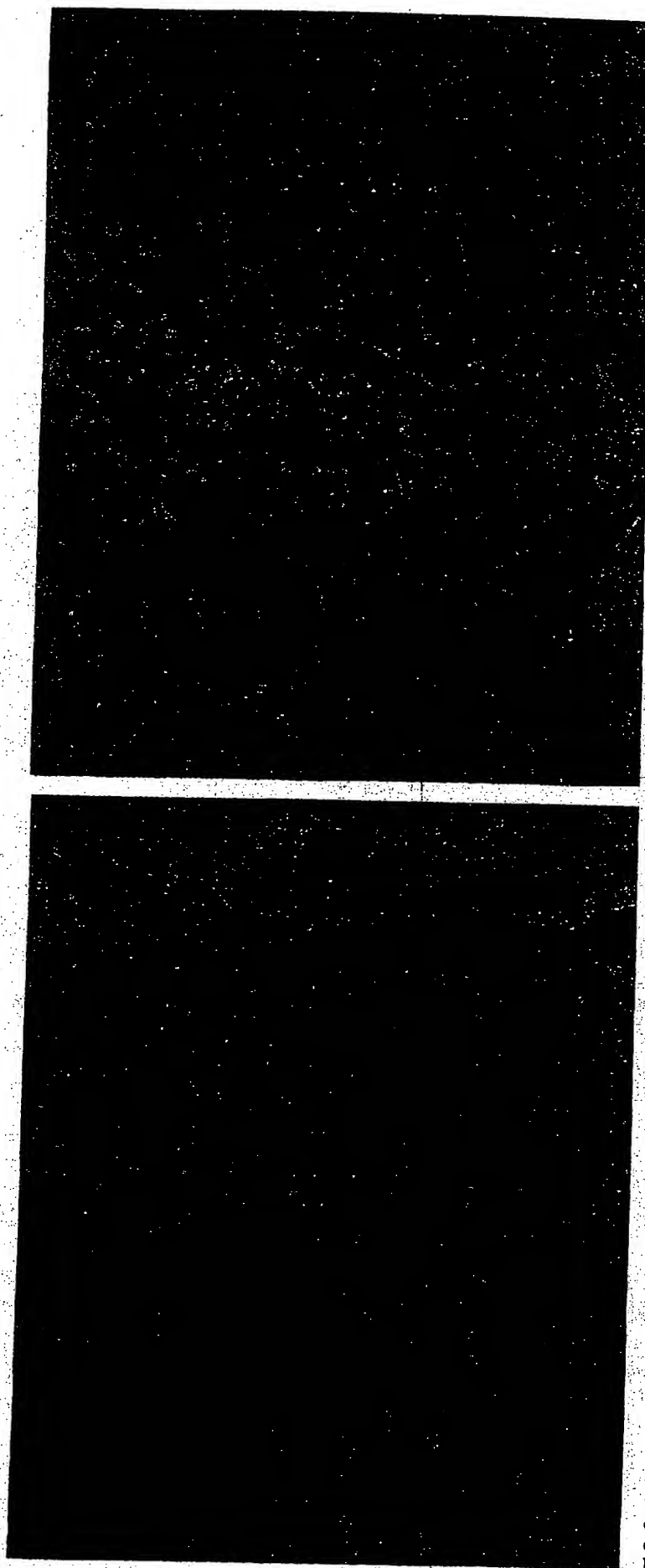


Fig. 2. In vivo transduction of rat hepatocytes with LX/ $\beta$ -Geo retrovirus. A 70% partial hepatectomy was performed on 3 to 4-week-old rats; 24 h later, either (A) 3.0 ml of LX/ $\beta$ -Geo retrovirus or (B) 3.0 ml of medium was infused into the portal vein. Seven days later, the liver was isolated, frozen, sectioned, and stained with X-gal and counterstained with nuclear fast red. Pictures represent sections from one of three rats infused with  $\beta$ -Geo, and one of one mock infused. Blue cells were only detected in the liver sections infused with LX/ $\beta$ -Geo retrovirus.

mine when to infuse retrovirus after partial hepatectomy for optimal transduction of rat hepatocytes, retrovirus was infused into rats at 0, 12, 18, 24, and 36 h after partial hepatectomy. In order to better quantitate the transduction efficiency and to ensure the blue cells were hepatocytes, we isolated and cultured the hepatocytes under conditions that selected for the growth of hepatocytes. Ten days after retroviral infusion, hepatocytes were isolated by collagenase perfusion and cultured overnight as described in Materials and Methods. Even if cell division occurs, the percentage of blue cells should not change, unless transduction affects hepatocyte growth. Twelve hours after X-gal staining, the percent transduction was calculated by scanning random fields at 400 $\times$  magnification and counting the number of blue cells and the total number of cells (at least 700). The optimal time for retroviral infusion appears to be 24 h after partial hepatectomy, at which time, 10–15% of the hepatocytes were transduced (Fig. 3).

**Retroviral Transduction Efficiency of Rat and Mouse Hepatocytes In Vitro.** Kay et al. (8) reported that using this in vivo retroviral transduction method in the mouse, they achieved a hepatocyte transduction efficiency of 1–2%, while we observed a 10–15% transduction efficiency in the rat. The 10-fold difference in transduction efficiency is not due to inherent differences in susceptibilities to infection by amphotropic retrovirus, since rat and mouse hepatocytes are transduced 20–25% with an amphotropic retrovirus in culture (14, 15, and Dr. M. Kay, personal communication). Thus both the rat and mouse hepatocytes are equally susceptible to amphotropic retroviral transduction.

**Histological Study of Regenerating Liver of Rat and Mouse.** Since both rat and mouse hepatocytes are equally susceptible to retroviral transduction in vitro, we investigated the regenerating livers of rats and mice. Perhaps, if the rat has a more vigorous hepatocyte replication response after partial

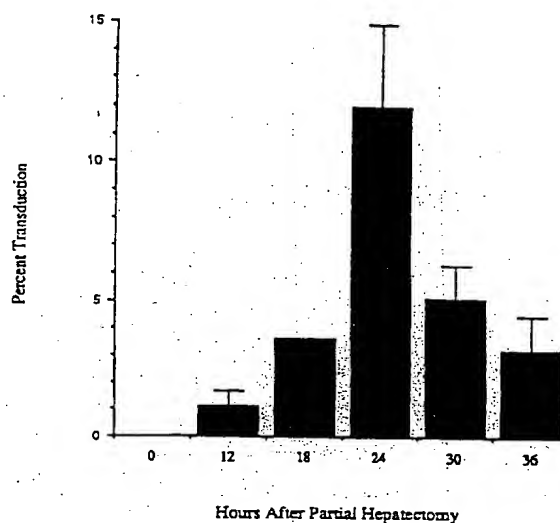


Fig. 3. Time course for in vivo infection of rat hepatocytes. The 70% partial hepatectomies were performed on 3 to 4-week-old male Lewis rats. Then at time 0 h ( $N = 2$ ), 12 h ( $N = 5$ ), 18 h ( $N = 1$ ), 24 h ( $N = 5$ ), 30 h ( $N = 4$ ) or 36 h ( $N = 3$ ) after partial hepatectomy, 3.0 ml of retroviral supernatant was infused into the portal vein. Seven to 10 days after transduction, the hepatocytes were isolated, cultured for 18 h, and stained with X-gal. Plates of hepatocytes were scanned at 400 $\times$  magnification and random fields were counted for the number of blue cells and the total number of cells. At least 700 cells were counted per time point. Bars represent mean and standard deviation. Range at 24 h is 8.6–16.7%.

hepatectomy than the mouse, it would explain the difference in transduction efficiency. The 70% partial hepatectomies were performed on rats and mice, and the remaining liver lobes were isolated at times when in vivo retroviral transduction is known to be optimal (24 h for the rat and 48 h for the mouse). Random fields were scanned at 400 $\times$  magnification and the number of mitotic figures and the total number of cells was determined. The mitotic index is the number of mitotic figures per 1000 cells (3). The rat liver had a mitotic index of 84.8, while the mouse liver had a mitotic index of 24.6. In both animals, the mitotic figures did not appear localized in any region of the liver (i.e., periportal).

**Expression of Human  $\alpha_1$ -Antitrypsin in Transduced Rat Hepatocytes.** In order to study the expression of retrovirally transduced

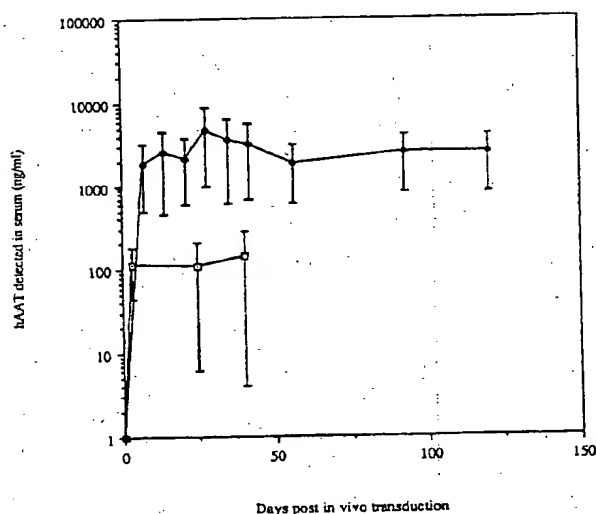


Fig. 4. Detection of human  $\alpha_1$ -antitrypsin in the serum of rats (closed squares) and mice (open squares) after in vivo transduction of hepatocytes with LX/hAAT retrovirus. The 70% partial hepatectomies were performed on mice and rats. The rats were infused 24 h later with 3.0 ml of supernatant containing the LX/hAAT retrovirus, while the mice were infused at 48 h with 1.0 ml of supernatant. Serum samples were collected from the animals at the indicated times and assayed for hAAT. The graph represents the mean  $\pm$  standard deviation for  $N = 4$  (mouse) and  $N = 7$  (rat).

genes long-term, we transduced rat hepatocytes with a retrovirus encoding the gene for human  $\alpha_1$ -antitrypsin (hAAT). hAAT was chosen because, being a secreted protein, its presence can be detected easily in the serum of the animal, and expression can be monitored long-term in each animal. hAAT has also been shown not to elicit an immunological response in dogs and mice when expressed from hepatocytes, but it can be detected and quantitated by an ELISA assay. A 70% partial hepatectomy was performed on rats, followed 24 h later by infusion of 3 ml of supernatant containing the LX/hAAT retrovirus. As can be seen in Fig. 4, hAAT can be detected in the rat serum seven days after retroviral transduction. The average levels reached 2000 ng/ml within a week after viral infusion and remained steady for at least 120 days. No hAAT was ever detected in the serum of rats transduced with a control retroviral vector (data not shown).

Mouse hepatocytes were also transduced in vivo with the same retrovirus under optimal conditions, and the presence of hAAT was monitored in their serum. hAAT levels in the mouse have also remained steady for 40 days of monitoring. As can be seen in Fig. 4, the average level of hAAT in the mouse of about 100–200 ng/ml is 10-fold lower than that in the rat. This observation is in agreement with the results from the  $\beta$ -gal staining showing that hepatocyte transduction in the rat is 10-fold higher than in the mouse.

## DISCUSSION

A 70% partial hepatectomy followed by infusion of retrovirus into the portal vein results in 1–2% hepatocyte transduction in the mouse (8), while in the rat, the same technique achieves 10–15% hepatocyte transduction. This difference is not due to differences in susceptibilities to retroviral transduction since both rat and mouse hepatocytes are equally transduced by amphotropic retrovirus in vitro. The difference is also not due to a greater number of virus particles being infused into the rat since we infused 3.0 ml into a 50-g rat (or  $6 \times 10^4$  PFU/g body weight) while Kay et al. (8) infused  $8 \times 10^4$  PFU/g into the mouse. The difference may be due, in part, to the fact that at the time for optimum transduction by retrovirus, the rat liver has a three to fourfold higher mitotic index than the mouse.

The percentage of transduced cells by our method (10–15%) is comparable to that achieved using the vascular isolation method (5–20%) of Ferry et al. (6) and 16% by Rozga et al. (7). A critical difference is that while the other groups' methods require very intricate surgical procedures to vascularly isolate the liver before infusion of retrovirus into the portal vein, our method only requires infusion of the retrovirus into the portal vein.

To study the expression of transduced

genes long-term, we used the secreted marker protein human  $\alpha_1$ -antitrypsin (hAAT). Using hAAT as a marker, one can study both the level of expression and changes in expression levels in one animal. This is superior to transducing several animals and sacrificing them at different times to determine percentage of cells still expressing the gene of interest, since one then introduces variability between animals. The amounts of hAAT being expressed varied from animal to animal. The levels ranged from 200 ng/ml to 4000 ng/ml. The levels of hAAT appear to be steady for at least six months. With respect to hAAT production, the mice produced up to 100–200 ng/ml of hAAT, while the rats averaged 2000 ng/ml. This is the expected result since 10-fold more hepatocytes are shown to be transduced by X-gal staining in the rat than in the mouse.

The focus of this study was to develop a simple and efficient method to deliver genes to the rat liver using recombinant retroviral vectors. We determined that a 70% partial hepatectomy followed 24 h later by infusion of 3 ml of retrovirus into the portal vein results in 10–15% hepatocyte transduction. Genes introduced in this manner are also expressed for at least six months. These results suggest that the rat is an excellent model for hepatic gene therapy of metabolic disorders.

#### ACKNOWLEDGMENTS

We thank Carol Toman for cloning, producing and titrating LX/hAAT, and Frances Leland for assaying the rat serum for hAAT levels. This work was supported in

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*Erratum*

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The name of one of the authors was inadvertently omitted by the authors from the paper, "Hepatic Gene Therapy: Efficient Retroviral Mediated Gene Transfer into Rat Hepatocytes In Vivo," Vol. 19, No. 5, 1993, pp. 491-497. The listing of authors should read Tadeusz M. Kolodka, Milton Finegold, Mark A. Kay, and Savio L.C. Woo.



COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

IN THE MATTER OF: Australian  
Patent Application 696764  
(73941/94). In the name of:  
Human Genome Sciences Inc.

- and -

IN THE MATTER OF: Opposition  
thereto by Ludwig Institute for Cancer  
Research, under Section 59 of the  
Patents Act.

Annexure GBC-17

This is **Annexure GBC-17** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

*Gary Baxter Cox*  
**Gary Baxter Cox**

WITNESS:

*Peytee K. McO*  
Patent Attorney

*PEYTEE K. MCO*

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LABORATORY INVESTIGATION

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# Methods in Laboratory Investigation

## A Simple, Quantitative Method for Assessing Angiogenesis and Antiangiogenic Agents Using Reconstituted Basement Membrane, Heparin, and Fibroblast Growth Factor

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JOSEPH A. HANEY, REBECCA R. PAULY, DERRICK S. GRANT, AND GEORGE R. MARTIN

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**BACKGROUND:** Blood vessel growth is necessary for normal tissue homeostasis and contributes to solid tumor growth. Methods to quantitate neovascularization should be useful in testing biological factors and drugs that regulate angiogenesis or to induce a vascular supply to promote wound healing.

**EXPERIMENTAL DESIGN:** An extract of basement membrane proteins (Matrigel) was found to reconstitute into a gel when injected subcutaneously into C57/BL mice and to support an intense vascular response when supplemented with angiogenic factors.

**RESULTS:** New vessels and von Willebrand factor antigen staining were apparent in the gel 2–3 days after injection, reaching a maximum after 3–5 days. Hemoglobin content of the gels was found to parallel the increase in vessels in the gel allowing ready quantitation. Angiogenesis was obtained with both acidic and basic fibroblast growth factors and was enhanced by heparin. Several substances were tested for angiostatic activity in this assay by coinjection in Matrigel with fibroblast growth factor and heparin. Platelet-derived growth factor BB, interleukin 1- $\beta$ , interleukin-6, and transforming growth factor- $\beta$  were potent inhibitors of neovascularization induced by fibroblast growth factor. Tumor necrosis factor- $\alpha$  did not alter the response but was alone a potent inducer of neovascularization when coinjected with Matrigel and heparin. Consistent with the previously demonstrated importance of collagenase in mediating endothelial cell invasion, a tissue inhibitor of metalloproteinases that also inhibits collagenases was found to be a potent inhibitor of fibroblast growth factor-induced angiogenesis.

**CONCLUSIONS:** Our assay allows the ready quantitative assessment of angiogenic and antiangiogenic factors and should be useful in the isolation of endothelial cells from the capillaries that penetrate into the gel.

**Additional key words:** Neovascularization, Matrigel

The development of a vascular supply is essential for the growth, maturation, and maintenance of normal tissues (1). It is also required for wound healing (2) and the rapid growth of solid tumors (3, 4) and is involved in various other pathological conditions (1, 5–7). Current concepts of angiogenesis, based in large part on studies on the vascularization of tumors (1), suggest that cells secrete angiogenic factors that induce endothelial cell migration, proliferation, and capillary formation. Although the factors that induce angiogenesis *in situ* are not well identified, numerous factors have been identified that induce vessel formation *in vitro* or *in vivo* in animal models. These include acidic fibroblast growth factor (aFGF) (1, 8, 9), basic (b) FGF (1, 9, 10), transforming

growth factor (TGF)- $\alpha$  (1), TNF- $\alpha$  (11, 12), vascular permeability factor or vascular endothelium growth factor (13–15), monobutyrin (16), angiotropin (17), angiotropin (18), hyaluronic acid degradation products (19) and age-associated glycosylation end-products (20). Also many compounds have been described as inhibitors of angiogenesis including a cartilage-derived inhibitor identified as tissue inhibitor of metalloproteinase (TIMP) (21, 22), platelet factor-4 (23), thrombospondin (24–26), laminin peptides (27), heparin/cortisone (28, 30), minocycline (31), fumagillin (32), difluoromethylornithine (33), and sulfated chitin derivatives (34).

The classic assessment of an angiogenic factor achieved either by embedding the factor in a controlled

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release polymer such as ethylene vinyl acetate or cellulose discs (reviewed in Ref. 22) and implanting the substances in the cornea of an animal's eye (35, 36) or by placing these substances on the chorioallantoic membrane of the chick embryo (37) and observing the sprouting of new vessels toward the pellet. Alginate-encapsulated tumor cells (38, 39) and gelatin-impregnated sponges (40) (Gelfoam, Upjohn, Kalamazoo, Michigan) have also been used as angiogenesis inducers. In the alginate tumor cell model, hemoglobin content was used to quantitate angiogenesis. In addition, several *in vitro* models have been used to examine the progression of angiogenesis including the sprouting, attachment, migration, invasion, and morphological differentiation of endothelial cells (8, 12, 41-44).

Here we report on a simple, rapid, and quantitative assay to assess inducers as well as inhibitors of angiogenesis. In brief, we inject a solution of basement membrane proteins supplemented with FGF and heparin subcutaneously in a mouse where it forms a gel. Sprouts from vessels in the adjacent tissue penetrate into the gel within days, connecting it with the external vasculature. Angiogenesis was quantitated by image analysis of vessels and by measuring the hemoglobin present in the vessels within the gel. This assay will facilitate the testing of both angiogenic and angiostatic agents *in vivo* and may allow isolation of the endothelial cells responding to the angiogenic factors for further studies *in vitro*.

## EXPERIMENTAL DESIGN

### PREPARATION OF ANGIOGENIC FACTORS AND VEHICLE

Liquid Matrigel maintained at 4°C was used as a vehicle to inject angiogenic factors subcutaneously into C57/BL mice. Various components were mixed with liquid Matrigel at 4°C which, when injected into a mouse, formed a single, readily recovered gel. Such gels were removed at various times and processed for histology, total protein, and hemoglobin content.

Matrigel, an extract of murine basement membrane proteins consisting predominantly of laminin, collagen IV, heparan sulfate, proteoglycan, and nidogen/entactin was prepared as a sterile solution as previously described (45). Heparin was dissolved in sterile phosphate-buffered saline (PBS) to 16,000 units/ml. Further dilutions were made with sterile filtered PBS containing 1 mg bovine serum albumin/ml. aFGF (HBGF-1) (R & D, Minneapolis, Minnesota) was diluted to 0.25 µg/ml with PBS/bovine serum albumin. Various amounts of heparin and/or FGF were mixed with 0.5-1.0 ml of Matrigel at 4°C in proportions not exceeding 1% of the volume of Matrigel to be injected. In some cases, other factors were included as noted.

### INJECTION AND PROCESSING OF GELS

C57BL mice (five per data point) were each injected subcutaneously with 0.5 ml Matrigel and 0-100 ng aFGF/µl and 0-64 units heparin/ml near the abdominal midline using a 25-gauge needle. The injected Matrigel rapidly formed a single, solid gel that persisted for at least 10 days in the mice. Mice were subsequently killed, and

gels were recovered and processed for further studies. Typically, the overlying skin was removed, and gels were cut out by retaining the peritoneal lining for support. For most histological sections, the skin and underlying peritoneum were Formalin-fixed immediately after dissection.

### QUANTITATION OF NEOVESSELS

Hemoglobin was measured using the Drabkin method (46) and Drabkin reagent kit 525 (Sigma, St Louis, Missouri). Samples for each point were from five different mice. The concentration of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel. Protein content of the supernatant fluid was determined using the BioRad protein assay method (47). The Optomax image analysis system (Optomax, Hollis, New Hampshire) was used for quantitation of histological specimens by light microscopy (see "Methods").

## RESULTS AND DISCUSSION

### MATRIGEL AS A VEHICLE FOR ANGIOGENIC FACTORS

In developing a more reproducible and quantitative angiogenic model, we utilized FGFs that are proven and potent inducers of neovascularization. When injected alone subcutaneously into mice, neither aFGF nor bFGF induced any visible signs of neovessel formation (data not shown). This is not unexpected since the factors would be expected to be rapidly cleared from the site. We tested Matrigel, a solution of basement membrane proteins isolated from the Engelbreth-Holm-Swarm tumor, as a vehicle for the slow release of angiogenic factors since it is a liquid at 4°C but forms a gel *in vivo*. Indeed, our studies showed that the gels which formed after subcutaneous injection of Matrigel alone were readily distinguished from surrounding tissue, persisted for at least 10 days, and produced little or no local reaction or angiogenic response (Fig. 1A).

Matrigel supplemented with FGF alone produced gels that showed a variable angiogenic reaction (data not shown). Magnitude of the angiogenic response was considerably greater in gels supplemented with both FGF and heparin (Fig. 1B and C). Subcutaneous injection of Matrigel plus aFGF and heparin at the ventral midline achieved optimal and reproducible responses, whereas material injected either anteriorly or posteriorly to the midline resulted in less consistent responses. Auerbach *et al.* (48) found similar regional differences in tumor growth that might also be related to the capacity for vascularization at these sites. Dorsal injections also induced consistent responses, but the abdominal location was used almost exclusively in this study. Gels could be recovered intact by dissection of the underlying peritoneum (Fig. 2). The tissue in contact with the FGF- and heparin-supplemented gels contained abundant and readily visible blood vessels (Fig. 2). Large neovessels were also present on the surface (Fig. 3A, B), whereas small, tortuous vessels were observed within the gel (Fig. 3C). The effect of age of the animal on the angiogenic

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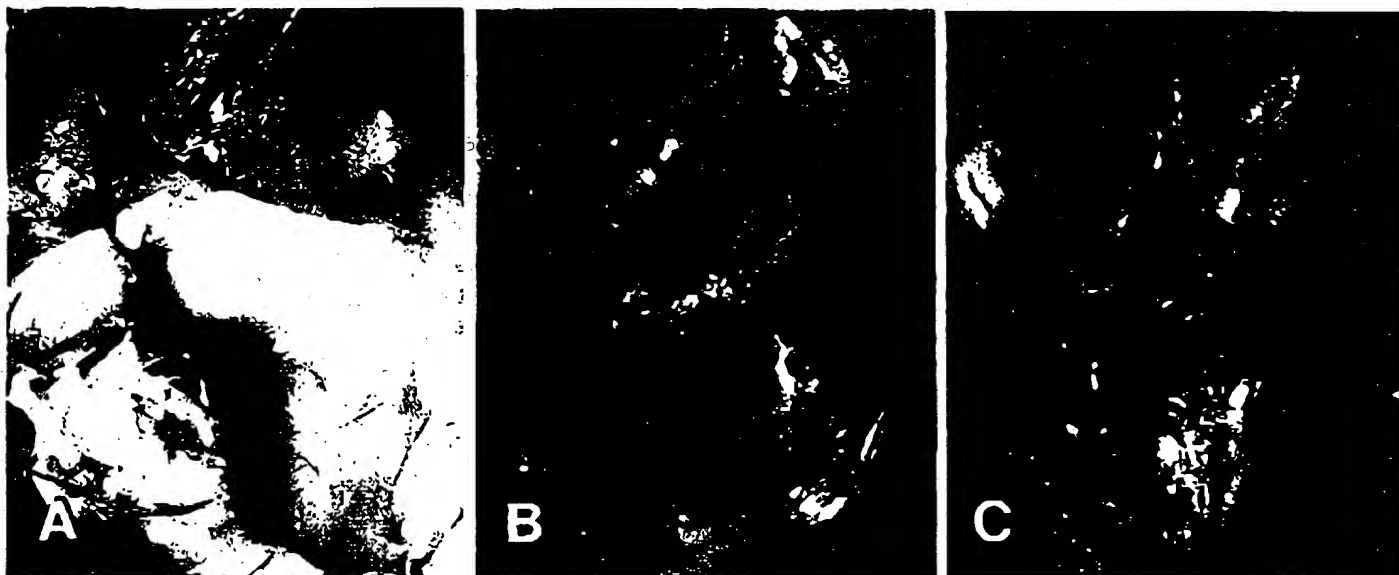


FIG. 1. Appearance of Matrigel gels on day 4 (A) or Matrigel supplemented with 1 ng/ml FGF and 40 units/ml heparin on day 1 (B) or day 4 (C) after subcutaneous injection. Overlying skin was removed to expose gels. Note that surface of gels as well as overlying skin flaps

contain many vessels. Bleeding seen here was also seen with some Matrigel/FGF and Matrigel/heparin injections, but these produced little or no vessel infiltration and was <10% of the amount of hemoglobin found in Matrigel/FGF/heparin gels at 4 days (see also Table 1).

16 U/ml

32 U/ml

48 U/ml

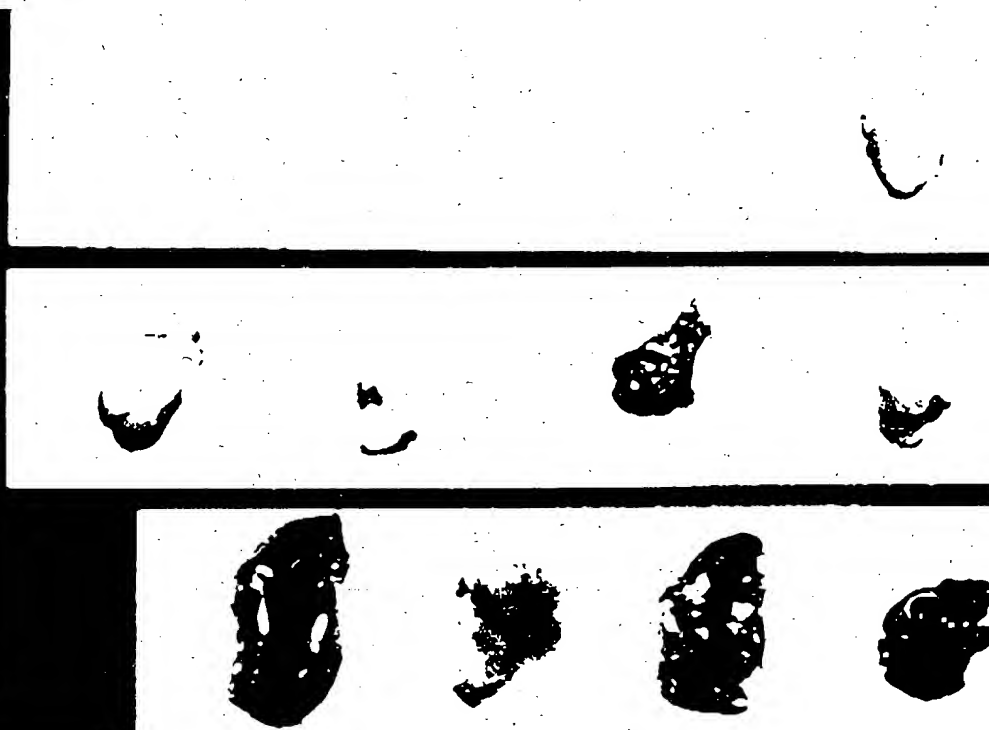


FIG. 2. Appearance of Matrigel gel recovered after 4 days *in vivo*: angiogenic response as a function of heparin concentration. Mice were injected with Matrigel and aFGF as in Figure 1C but with various heparin doses. After killing animals, skin was removed, and gels were

cut out with intact peritoneal lining for support and placed on tissue culture dishes for photography. Each gel was between 0.8 and 1.4 cm long. Heparin dependence of response is apparent (see also Table 1).

response showed that vessel formation was reduced in young (6 month) animals compared with older mice (12, 18, or 24 months of age) where the response was typically twice as strong.

#### HISTOLOGY AND ENDOTHELIAL CELL STAINING

Sections examined with the Trichrome-Masson stain (Fig. 4) showed that cells invaded the gel within 24 hours and persisted for up to 8 days with a progressive increase

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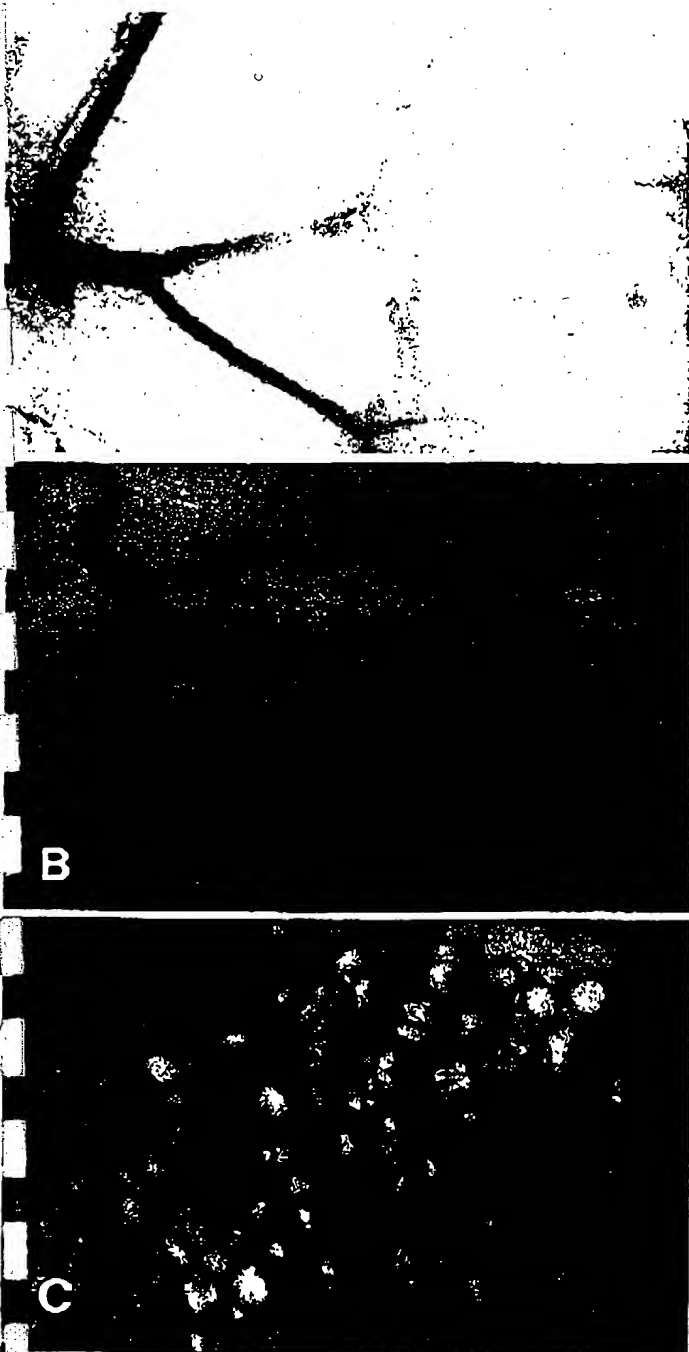


FIG. 3. Appearance of vessels associated with FGF/heparin-supplemented gels. Vessels surrounding gel appear to derive from peritoneal lining (A) and skin (B). Arrows, regions in injected gel that contain tubular neovessels derived from ramified pre-existing vessels. Small tubular tubes are also prevalent inside gel (C, arrows) that appears atypical.

linear structures containing red blood cells which was indicative of functional vessels. Sections of the gel were reacted with antibody to factor VIII antigen (von Willebrand factor) to confirm the presence of endothelial cells in association with the vessels. The presence of capillary-sized vessels in the gel was apparent at 72 hours (Fig. 5).

These neovessels were also apparent by 48 hours (not shown) and are smaller than other factor VIII positive structures (pre-existing vessels) on the periphery of the Matrigel (Fig. 5, arrowheads). Neovascularization was not observed at 24 hours, although inflammatory cells were observed in the region between the Matrigel and skeletal muscle.

#### QUANTITATION OF FGF-INDUCED ANGIOGENESIS

The increase in vessels in the gels, based on specific von Willebrand factor stain as quantitated by an image analysis system (Fig. 6), was similar to the increase in cells (hematoxylin/eosin and trichrome stain). Measurement of hemoglobin content indicated formation of a functional vasculature at the site of angiogenesis. As judged by hemoglobin content, the angiogenic response to FGF was time dependent, clearly visible by days 1-2, reached a plateau by days 3-4, and persisted through day 8 (Fig. 6, lower panel) occurring with similar kinetics as observed for the accumulation of neovessels (Fig. 6, upper and middle panels).

In the presence of heparin (64 units/ml), the maximal angiogenic response occurred at 1 ng/ml aFGF (Table 1) followed by a decrease and then a subsequent increase at higher levels of FGF. These data are consistent with the down-regulation of FGF receptors in the presence of higher levels of the growth factor (57). In some experiments, higher doses of FGF were used (125 and 250 ng FGF/ml), and these showed responses similar to those observed at 100 ng/ml. In contrast, heparin induced a linear increase in angiogenesis in the presence of 1 ng/ml FGF (Table 1). The lowest concentration of heparin that resulted in consistent vascularization of the gels was 40 units/ml. At this concentration of heparin, the FGF response was also biphasic with an optimum again at 1 ng/ml (data not shown). The amount of aFGF (0.5 ng) required for an angiogenic response in these assays is similar to the levels of FGF necessary for endothelial cell growth in culture (49, 50) and to the levels required to elicit an angiogenic response in the chick allantoic membrane (10). Our results suggest that the angiogenic response induced by heparin and aFGF occurs at physiologically relevant doses of FGF observed previously using other assays and other angiogenic factors.

#### INHIBITORS AND ACTIVATORS OF NEOVASCULARIZATION

We tested several cytokines for angiogenic activity in this assay in the presence of heparin to determine whether the assay was comparable to other established angiogenesis assays (Table 2). Of the various factors tested, aFGF, bFGF, and TNF- $\alpha$  induced an angiogenic response (Table 2), consistent with previous reports on these factors (1, 11, 51) whereas TGF $\beta$ , PDGF, interleukin (IL)-1, and IL-6 were inactive.

We also assessed the angiostatic activity of certain cytokines when included with aFGF (Table 3). IL-1 $\beta$ , IL-6 (52), and TGF $\beta$  inhibited the angiogenic response to aFGF. The TGF $\beta$  dose response showed inhibition at concentrations as low as 0.2 ng/ml. PDGF BB was also

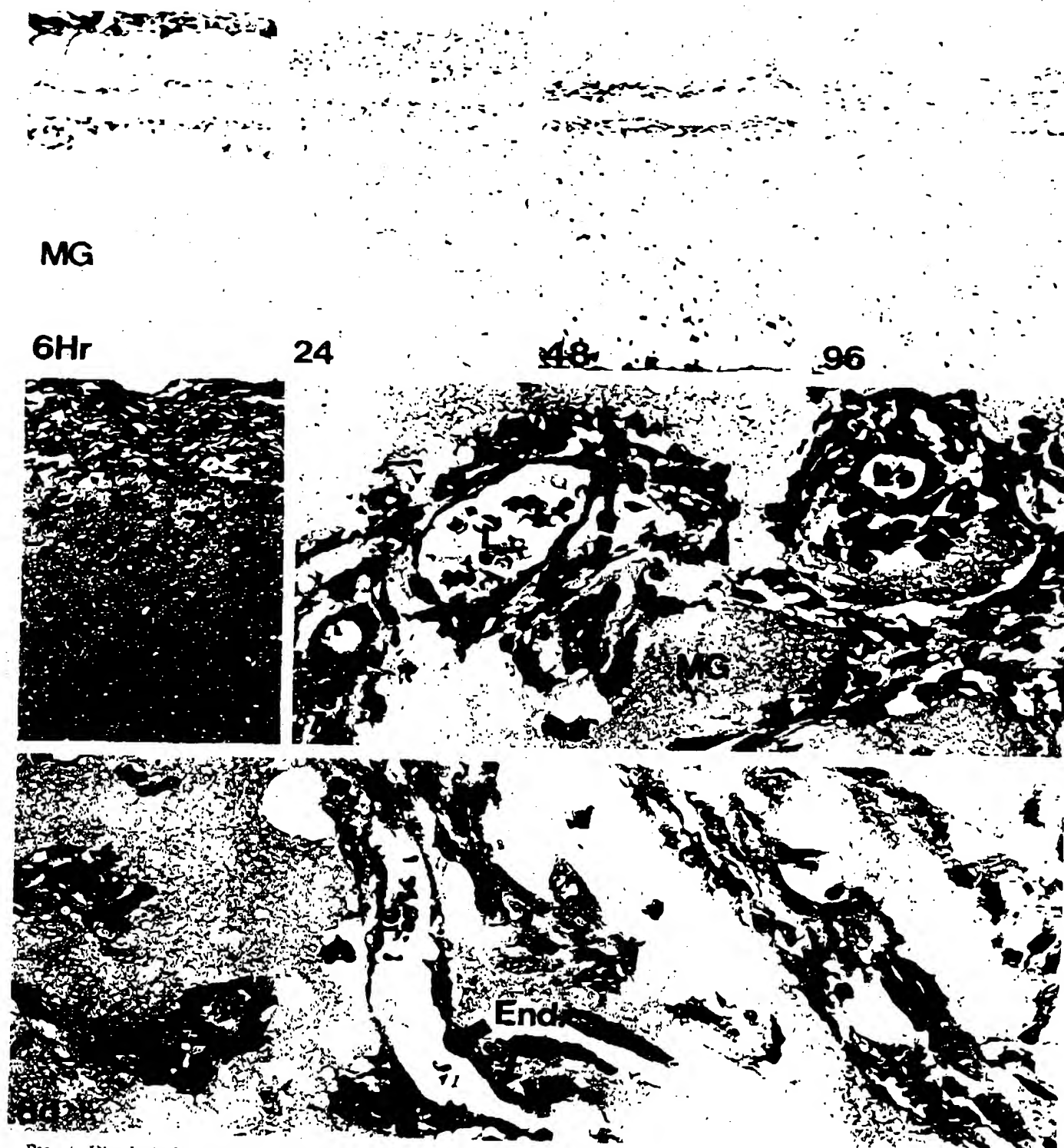


FIG. 4. Histological analysis of recovered gels: course of vessel formation. Samples were prepared for histology as described in "Methods" at different times after injection of Matrigel containing FGF and heparin. Trichrome-Masson stained specimens show the progressive invasion of cells into the Matrigel (MG) over 6, 24, 48, and 96 hours (top four panels; Mag =  $\times 125$ ). By 7 days, there is more organization

of the cells into linear structures. At higher magnification ( $\times 500$ ; middle panel, 7 day), the connective septa within the Matrigel exhibits large blood vessels from which an extension of a vessel into the Matrigel is evident (two arrows). At 6 days, many vessels within the Matrigel are well formed exhibiting a clear endothelium (End).



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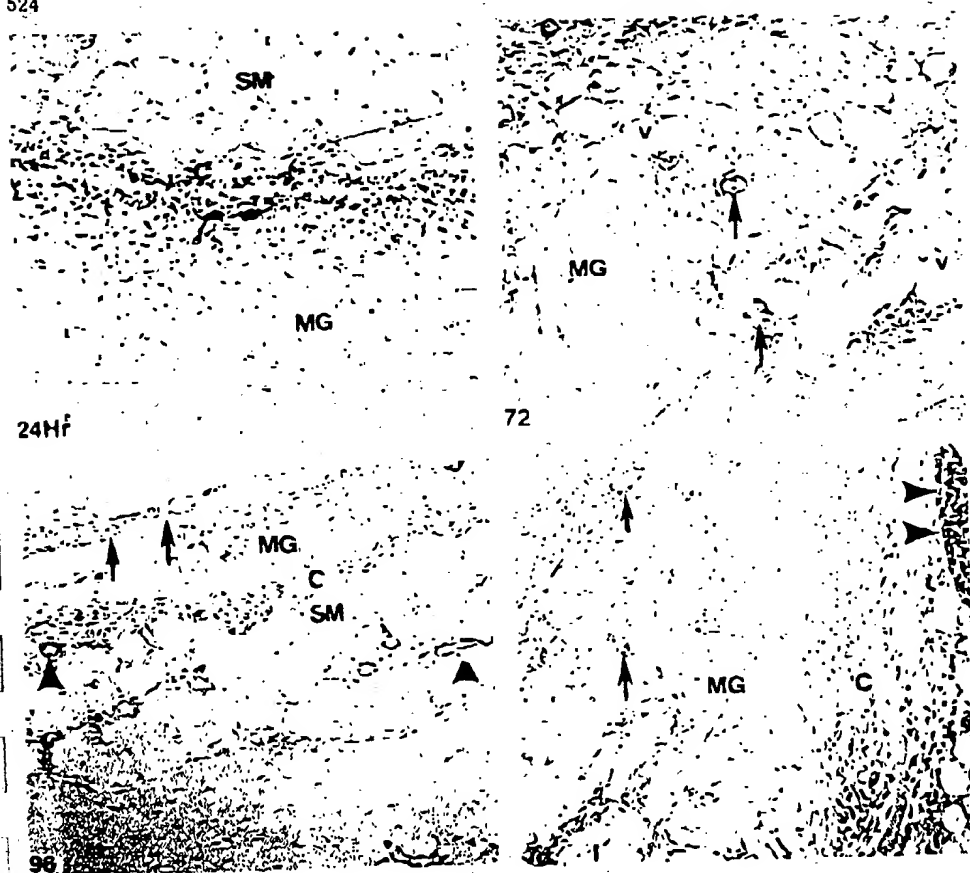


FIG. 5. Factor VIII staining of neovessels. Gels recovered after 1, 3, 4, and 7 days were stained with von Willebrand factor antibody as described in "Methods." Presence of neovessels (arrows) in Matrigel (MG) layer can be distinguished from existing vessels (arrowheads) near skeletal muscle (SM) and collagen (C) interface. Small vessels (72 hours), horizontally coursing structures (96 hours), and ramifying blood vessels (7 days) are noted. Vacuoles (v);  $\times 40$ .

a potent inhibitor probably acting indirectly since endothelial cells do not express a receptor for this factor (53).

TIMP, a collagenase inhibitor (21), is also found in cartilage (22) where it may maintain cartilaginous tissue in an avascular state by inhibiting endothelial cell migration (22). Addition of recombinant 0.5 mg/ml TIMP in the Matrigel/heparin/FGF mixtures showed essentially complete inhibition of neovascularization at day 3 as measured both by hemoglobin content (Fig. 7) and by examination of the gel for infiltrating vessels (not shown). These observations are consistent with the known role of metalloproteases in the invasion of endothelial cells through basement membrane (43) and for the role of metalloproteases in angiogenesis (22).

## DISCUSSION

We have developed a quantitative angiogenesis assay based on the ability of an extract of basement membrane proteins (Matrigel) to form a solid gel when injected into mice and to support a rapid and intense angiogenic reaction in the presence of FGF and heparin. Matrigel, while stimulating cell attachment and morphogenesis when used as a substratum in tissue culture, does not induce an angiogenic response *in vivo* alone. Matrigel has been found to promote the differentiation of endothelial cells into capillary-like structures in culture (12, 41) and when used as a vehicle *in vivo* may enhance the selectivity of endothelial cells entering the gel since

basement membranes are not readily crossed by fibroblasts and certain other cells.

Gels supplemented with FGF and heparin induced intense vascularization. Numerous large vessels were apparent on the surface of the gel, whereas vessels within the gel were smaller and more tortuous. Vessel formation was quantitated by measuring the hemoglobin present in the dissected gels and confirmed by histological staining for von Willebrand factor and with Trichrome-Masson stain. Vessel formation was apparent as early as 2 days, reached a plateau after 4 days, and persisted up to 8 days. Maximal and consistent responses required both FGF and heparin, and distinct concentrations of each factor were required for optimal responses. The site of injection and age of the animal affected magnitude of the response.

The correlation of hemoglobin content with vessel formation was previously described using alginate-entrapped tumor cells to elicit angiogenesis *in vivo*. Factor VIII-stainable vessels were found to correlate with hemoglobin content and pooling of radiolabeled red blood cells at the alginate injection site (38). The requirement for heparin with FGF in angiogenesis assays (54) and fibroblast growth and differentiation (55, 56) appears to be due to both a stabilization of FGF and conformational changes in FGF required for receptor binding (57). Heparin also enhances the angiogenic activity of factors produced by 3T3 adipocytes (58), recently shown to be mediated by monobutyryl (16). In our assays, aFGF was

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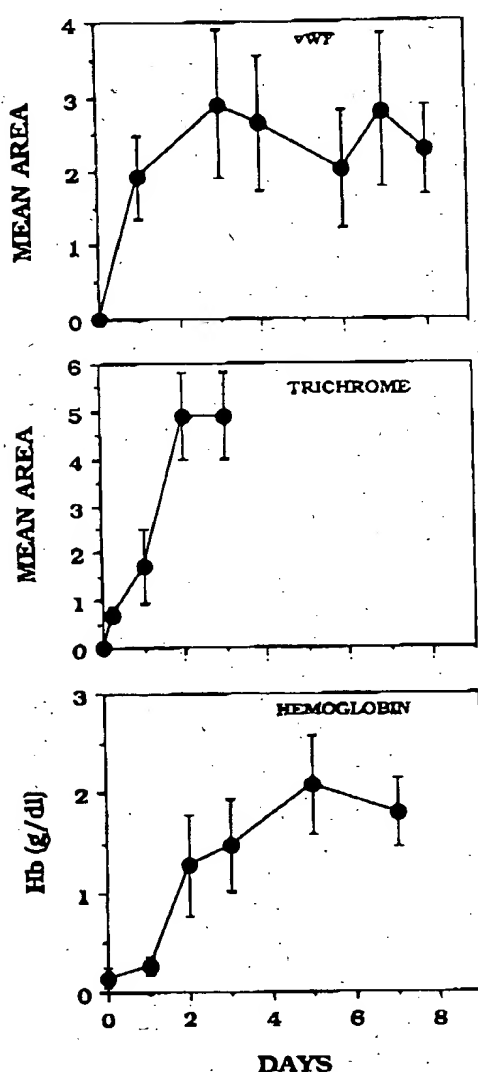


FIG. 6. Quantitation of neovascularization. Histological slides were examined with an Optomax image analysis system, and mean area in a 20X or 40X field was quantitated for slides stained with von Willebrand factor antibody (vWF) or Trichrome-Masson stain (Trichrome). Each point represents mean area per field ( $\times 10^4 \mu m^2$ ) of 10-20 fields, and error bars are for standard deviation from mean. Hemoglobin measurements at these time points were determined as described in "Methods" and Table 1. Data represent the mean hemoglobin values from at least five mice per point with SEM as indicated.

potent at concentrations reported previously to be effective in both *in vivo* and *in vitro* assays. The course of the response was also comparable to results obtained with FGF in other assays and similar to that reported for other angiogenic agents like angiotropin (17). Heparin was required for angiogenesis in our assay even though heparan sulfate is present in the Matrigel possibly because the amount of heparan sulfate in the Matrigel is relatively low compared with normal basement membrane (59) and since FGF remains bound to heparan sulfate proteoglycan until released by enzymes (60). Not unexpectedly, the angiogenic response to FGF occurred more rapidly than the response observed with alginate-

TABLE 1. ACIDIC FGF DEMONSTRATES A BIPHASIC ANGIOGENIC RESPONSE THAT IS ENHANCED BY HEPARIN

	Hemoglobin <sup>a</sup> g/dl
aFGF (ng/ml) <sup>a</sup>	
0	0.28 $\pm$ 0.36
0.1	1.00 $\pm$ 0.45
1.0	3.20 $\pm$ 2.20
10	0.23 $\pm$ 0.15
100	0.94 $\pm$ 1.18
Heparin (units/ml) <sup>c</sup>	
2.0	ND <sup>d</sup>
6.0	0.04 $\pm$ 0.03
20	0.15 $\pm$ 0.20
32	0.16 $\pm$ 0.49
40	0.69 $\pm$ 0.60
64	2.72 $\pm$ 1.00

<sup>a</sup> Matrigel gels contained 64 units heparin/ml and were processed 3 days after injection.

<sup>b</sup> Values ( $\pm$ SE) are averages of at least five animals.

<sup>c</sup> Matrigel contained FGF at 1 ng/ml for each experiment.

<sup>d</sup> ND, Not detectable.

<sup>e</sup> Heparin at 40 units/ml was the lowest concentration to yield consistent vessel formation.

TABLE 2. DETECTION OF ANGIOGENIC ACTIVITY USING VARIOUS NEOVASCULARIZATION FACTORS

Factors Added to Matrigel + Heparin	Hemoglobin g/dl
None	0.10 $\pm$ 0.02
aFGF (1 ng/ml)	1.30 $\pm$ 0.07 <sup>a</sup>
TGF $\beta$ (20 ng/ml)	0.06 $\pm$ 0.02
PDGF BB	
2 ng/ml	0.10 $\pm$ 0.06
20 ng/ml	0.15 $\pm$ 0.08
200 ng/ml	0.07 $\pm$ 0.03
PDGF AB (5 units/ml)	0.11 $\pm$ 0.04
IL-1 $\beta$ (1 ng/ml)	0.22 $\pm$ 0.02
IL-6 (10 ng/ml)	0.18 $\pm$ 0.05
bFGF	
1.0 ng/ml	0.14 $\pm$ 0.13
10 ng/ml	0.20 $\pm$ 0.17 <sup>a</sup>
100 ng/ml	0.86 $\pm$ 0.70
TNF $\alpha$ (10 ng/ml)	2.30 $\pm$ 2.00 <sup>a</sup>

Matrigel (0.5 ml) and heparin (40 units/ml) were mixed with various factors and injected subcutaneously. Responses were quantitated 4 days later. TGF $\beta$ , PDGF BB, IL-6, IL-1 $\beta$ , or PDGF AB did not induce neovascularization.

<sup>a</sup> Acidic FGF, basic FGF, or TNF $\alpha$  were potent inducers of angiogenesis.

encapsulated tumor cells (38), which presumably require some time to generate their own factor(s). A related angiogenic factor, vascular permeability factor (13), has been shown to induce vascular permeability *in vivo* at 8 ng/animal and is active between 0.1 and 2 ng/ml as a mitogen for endothelial cells *in vitro*. In addition, the vascular permeability factor induces angiogenesis in the rat corneal assay at 20 ng (13). An unrelated chemical inducer of angiogenesis, monobutyryl (16), has been shown to be angiogenic in the chorioallantoic membrane



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TABLE 3. INHIBITION OF ANGIOGENESIS BY PDGF, IL-1 $\beta$ , IL-6, AND TGF- $\beta$ 

Factors Added to Matrigel + Heparin + aFGF	Hemoglobin g/dl
None	1.30 $\pm$ 0.07
TNF $\alpha$ (10 ng/ml)	1.10 $\pm$ 1.10
TGF $\beta$	
0.02 (ng/ml)	1.70 $\pm$ 1.50
0.2 (ng/ml)	0.08 $\pm$ 0.05
2.0 (ng/ml)	0.15 $\pm$ 0.13
20 (ng/ml)	0.24 $\pm$ 0.25
PDGF BB (200 ng/ml)	0.16 $\pm$ 0.07
IL-1 $\beta$ (1 ng/ml)	0.14 $\pm$ 0.10
IL-6 (10 ng/ml)	0.17 $\pm$ 0.12

Gels contained aFGF (1 ng/ml) + heparin (40 units/ml) and various cytokines. Hemoglobin levels in the gels are shown after 4 days. TNF $\alpha$  and TGF- $\beta$  had no effect on the angiogenic response. PDGF BB, IL-1 $\beta$ , IL-6, and TGF- $\beta$  inhibited the response.

### Angiogenesis: TIMP (1 ng FGF/ml; 64 U Hep/ml)

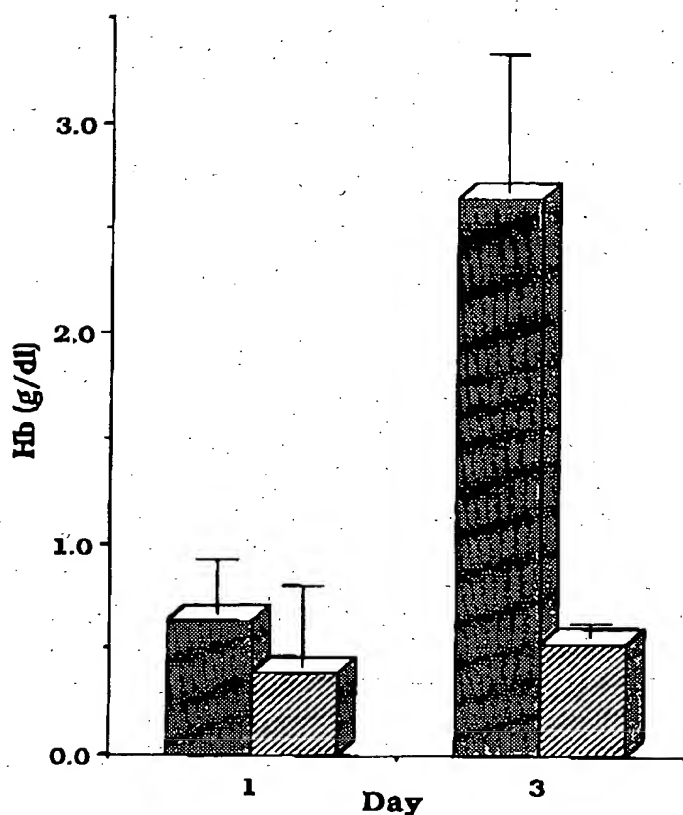


FIG. 7. Inhibition by TIMP: TIMP (collagenase inhibitor) is a potent inhibitor of aFGF-induced angiogenesis. Recombinant TIMP protein (0.5 mg/ml) was included with Matrigel (0.5 ml), FGF (1 ng/ml), and heparin (64 units/ml) at injection (hatched bars). Gels from at least five animals per point were analyzed after day 1 or 3. Shown for comparison are hemoglobin levels in gels that contained Matrigel, FGF, and heparin but lacked TIMP (solid bars).

assay at 20 pg (0.14 pmol), whereas aFGF in our assays is active at 0.025 pmol.

Other cytokines were tested in this assay including IL-1 $\beta$ , IL-6, and TGF- $\beta$  and these were found to be potent inhibitors. IL-6 enhances production of TIMP (61), which may inhibit collagenase and endothelial cell migration (62). TGF $\beta$  inhibits endothelial cell proliferation and migration (63), although it exhibits angiogenesis *in vivo* in some assays (64). We have measured the content of TGF $\beta$  in the Matrigel to be 8–14 ng/ml dependent on batch. However, all TGF $\beta$  is in the latent form, and we cannot detect any active TGF $\beta$  in the preparations using the CCL64 mink lung epithelial cell bioassay that measures inhibition of proliferation of CCL64 cells by active TGF $\beta$ . Therefore the observed results with exogenous TGF $\beta$  (active form) reflect activity of the added factor. IL-1 has been shown to regulate endothelial cell growth via autocrine mechanisms (65) that may lead to programmed cell death (apoptosis) as is observed in endothelial cells deprived of FGF (66). TNF $\alpha$  and bFGF induced neovessel formation. TNF $\alpha$  has been shown to activate macrophages (51) that in turn produce angiogenic factors.

In summary, the advantages of the assay presented here are that it is rapid, reproducible, quantitative, and does not require a surgical procedure for implantation. It allows detection of both angiogenic and anti-angiogenic factors and may allow isolation of those endothelial cells that penetrate into the gel. We have also used this system to assess the capacity of mice of different ages to initiate an angiogenic response, and this type of study would be of interest in both hypertensive and diabetic mice. Such systems may be useful in identifying and isolating biological factors and drugs able to regulate angiogenesis. In addition, the potential exists to induce an additional vascular supply in wounded or ischemic tissue where it is needed to restore normal healing and regeneration.

## METHODS

### ANIMALS, CELLS, AND GROWTH FACTORS

Female C57Bl/6 mice (Jackson Laboratories, Bar Harbor, Maine) were used at 6–8 weeks of age. Heparin was obtained from Gibco/Bethesda Research Laboratories. Bovine aFGF (HBGF I) bFGF (HBGF II) and TGF- $\beta$  isolated from human platelets were from R & D Systems. Recombinant TNF- $\alpha$  was a generous gift from Dr. John Isaacs (Johns Hopkins University) and was originally obtained from Cetus Corporation. PDGF and recombinant IL-6, were from Collaborative Research (Bedford, Massachusetts). IL-1 was a kind gift from Dr. Nigel Waite at Upjohn. TIMP was a gift from Dr. David Carmichael (Synergen, Boulder, Colorado). CCL64 mink lung epithelial cells were obtained from American Type Culture Collection (Rockville, Maryland).

### PREPARATION OF BASEMENT MEMBRANE MIXTURES

Reconstituted basement membrane (Matrigel) was prepared from the Engelbreth-Holm-Swarm tumor as described (45), sterilized by dialysis against chloroform, and stored at  $-20^{\circ}\text{C}$ . Before use, Matrigel was thawed at  $4^{\circ}\text{C}$  and placed immediately on ice before addition of aFGF, heparin, or other growth factors.

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Matrigel prepared by standard methods consists of 5–10 mg protein/ml and yields reproducible results in the angiogenesis assay. The commercial source of heparin (Gibco/Bethesda Research Laboratories) is critical and only batches yielding consistent results were used.

## HISTOLOGY AND FACTOR VIII RELATED ANTIGEN STAINING

All specimens were fixed in 10% buffered Formalin for at least 24 hours, progressively dehydrated in increasing percentages of ethyl alcohol (70, 80, 95, 100, and 100%), cleared in Histoclear, embedded in paraffin under vacuum, sectioned at 5  $\mu$ m thickness, deparaffinized, and stained with Harris hematoxylin and eosin (67).

Selected specimens were also stained for Factor VIII-related antigen using an immunoperoxidase method (68) or Trichrome-Masson (69). Briefly, 5- $\mu$ m sections were placed on silanized slides, dried overnight at 64°C, deparaffinized, hydrated, and placed into 3% hydrogen peroxide to quench endogenous peroxide. After rinsing in deionized water, the slides were enzymatically treated with 0.05% Pronase (Calbiochem, San Diego, California) in PBS with 0.114% EDTA at 37°C for 20 minutes. Enzyme activity was then abolished with 95% ethanol for 5 minutes. After PBS rinsing, rabbit anti-human von Willebrand Factor antibody (Dako, Carpinteria, California) diluted 1:1000 in 0.05% nonfat dry milk in PBS was applied to the slides that were placed in a humidity chamber overnight at 4°C. After rinsing in PBS the next morning, test slides were incubated at room temperature for 20 minutes in biotinylated antirabbit IgG (Vector, Burlingame, California) diluted 1:1000 in PBS with 0.5% nonfat dry milk. Nonimmune goat serum (5% v/v) was added to block nonspecific staining. Slides were then rinsed in three changes of PBS, incubated for 20 minutes in horseradish peroxidase conjugated streptavidin (Jackson ImmunoResearch, West Grove, Pennsylvania), diluted 1:1500 in PBS with 0.5% nonfat dry milk, rinsed in tap water, dried, mounted in Crystal Mount, dried at 80°C for 20 minutes, and coverslipped with Permount.

## IMAGE ANALYSIS AND NEOVESSEL QUANTITATION

To measure the total area of neovessels, a computerized digitalizer, the Optomax image analysis system (Optomax), was used. This system consists of a high sensitivity CCTV camera mounted on a Nikon Optiphot-2 microscope. The image is displayed on a color video monitor that is interfaced with a microprocessor. Histological slides stained with von Willebrand factor antibody or Trichrome-Masson stain were examined by adjusting the color contrast to enhance the specifically stained vessels. The mean area per field ( $\times 10^6 \mu\text{m}^2$ ) from 10–20 fields (20 $\times$  or 40 $\times$ ) was calculated with standard deviation from the mean. The vascularized area to be measured was chosen for its proximity to the skeletal muscle/collagen interface from which the neovessels originated before entering the Matrigel.

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COMMONWEALTH OF AUSTRALIA

*(Patents Act 1990)*

IN THE MATTER OF: Australian

Patent Application 696764

(73941/94). In the name of:

Human Genome Sciences Inc.

- and -

IN THE MATTER OF: Opposition

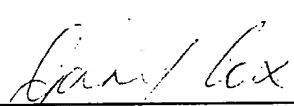
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Annexure GBC-18

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Thirteenth day of December 2000.

  
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**Gary Baxter Cox**

WITNESS:

  
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Patent Attorney

PEYTEE KHD

Meth and Pind (Am J Clin Pharmacol 1983; 104): 221-226

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## Angiogenesis: Quantitative Assessment by the Chick Chorioallantoic Membrane Assay

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### SUMMARY

The aim of the present work was to improve quantitative assessment of angiogenesis by chick chorioallantoic membrane (CAM) assay based on measurement of DNA synthesis. Following incubation of Pflithyridine (3H-T) with CAM in vivo, incorporation of 3H-T to DNA fraction was expressed as percent of total 3H-T present in the initial homogenate of CAM, regardless of CAM weight and full recovery of applied radioisotope. The assay required simple, partial isolation of DNA to remove traces of free or unspecifically bound 3H-T. These modifications gave a reproducible assay with adequate precision (10-20%). DNA content of CAM did not change between 10-15 days of embryo development and growth of CAM was completed on day 11. 10 to 12-day old CAMs were used for evaluation of the assay. Using a qualitative approach (visual scoring), assays of several tumors were implanted on CAM. Experiments of tumors that produced the highest were stimulated DNA synthesis in CAM. A similar effect was induced by EGF while cytostatics inhibited DNA synthesis in CAM. Since stimulation of angiogenesis is not a cell type-specific phenomenon, the assay in the present modification should be the studies on angiogenic factors. With the help of this assay the angiogenic activity of dexamethasone of human endometrium was described.

Key words: Chorioallantoic membrane - Angiogenesis - DNA - EGF - Carcinoma endometrium

### INTRODUCTION

The chorioallantoic membrane (CAM) of the chick embryo has been introduced by Folkman (13) and his colleagues to assay the angiogenic activity of various tumors, normal tissues, and cells (1, 4, 8, 13). Assays on CAM constitute a large-scale screening test for substances that promote or inhibit vascular growth (6, 7, 12, 15). Despite such wide use, the CAM assay has limitations, such as the problems of differentiation of newly formed vessels from the hyperemic response or from the mechanical effect of implant and the problems related to the immune reactions emerging on day 15 of embryo development (4, 5, 6, 18, 22).

Several procedures were introduced to optimize visual assessment of vascular response of CAM, originally relying on the arbitrary scale (13, 21). These include: shell-less culture of chick embryo (2, 10), estimation of vascular density within superimposed circle (6, 11), the calculation of coefficient of angiogenesis (28), and the use of automatic image analyzer (26). However, all these procedures still suffer from subjectivity of evaluation.

Recently, a very simple method, based on the estimation of DNA synthesis, has been used by Thompson *et al.* (27) for quantitative assessment of the angiogenic response on CAM. However, this method is suitable only when pooling (up to five) of CAMs is possible as the low precision of assay for individual eggs exceeds 40% (27). For large-scale screening of angiogenic activity the assay for individual eggs is required. We, therefore, modified accordingly the original method of Thompson *et al.* (27) and the present results show that following modification, this simple assay can be used to monitor quantitatively angiogenic activity on individual CAMs.

### MATERIALS AND METHODS

#### Visual evaluation of CAM response

Fertile hens' eggs (Astra, Sweden), washed in water and 0.8% chlorhexidine in 70% ethanol were placed in a humidified incubator at 37°C. On day 4, following removal of 0.5 ml of albumen, the window was made in the shell over an air space to expose CAM.

On day 10 of embryo growth, in aseptic conditions, slices (2x2x1 mm) of meningioma, adenocarcinoma of endometrium or normal endometrium (obtained immediately following surgery from the Departments of Neurosurgery or Obstetrics and Gynecology, District Hospital, Rzeszów) were placed directly on CAM or separated from it by a sterile Millipore filter, 0.45  $\mu$ m (Millipore Corp., Bedford, Mass, USA). During further incubation for 5 days the vessels of CAM were examined each day under a stereomicroscope in a blind fashion and the score according to the arbitrary scale (13) was assigned. Each tissue was implanted on six CAMs and median of CAM maximal scores (occurring usually on day 15) for each clinical specimen was recorded. The frequencies for each scoring interval (see Results) were tabulated and the chi square test was used.

#### Estimation of DNA synthesis

The entire procedure of Thompson *et al.* (27) was modified. Methyl-[ $^3$ H]thymidine (specific activity: 87 Ci/mmol, Amersham, UK), usually at a dose of 2.0  $\mu$ Ci in 0.2 ml of phosphate buffered saline (PBS), was incubated with CAM *in vivo* in most experiments for 2 hr. Following incubation the eggs were placed at  $-20^{\circ}\text{C}$  for 30 min. The CAM was cut out, rinsed thoroughly in ice cold PBS, weighed and homogenized (Polytron, MPW-309) in 3 ml of water for 5 min at  $4^{\circ}\text{C}$  and disintegrated for 30 sec at  $4^{\circ}\text{C}$  in the Ultrasonic Disintegrator (UD-11). During mixing, 0.1 ml of the homogenate was removed for calculation of the total radioactivity in dpm. Homogenate (2 ml) was then mixed with 5 ml of ice-cold 5% perchloric acid. The precipitate obtained after centrifugation (2500xg, 10 min  $4^{\circ}\text{C}$ ) was washed twice with 5 ml of 5% perchloric acid. The precipitate was then shaken and washed twice (each time with centrifugation step, as above) successively with 5 ml of the following solvents: 96% ethanol, 96% ethanol-ethyl ether mixture (4:1 v/v), and ethyl ether. Following evaporation of ether, the precipitate was treated with 5 ml of 5% perchloric acid and heated to  $96^{\circ}\text{C}$  for 15 min. After centrifugation the supernatant was divided. 0.5 ml of supernatant was neutralized (with 0.1 M NaOH) and the radioactivity was estimated in a Beckman LS-7000 scintillation counter using II number for quenching determination. In the second part of the supernatant, DNA was measured colorimetrically (17) following addition of diphenylamine and with the help of Specol colorimeter. The radioactivity measured (in dpm) in the fraction containing DNA was expressed in percent of total radioactivity of the homogenate. Percent of incor-

porated thymidine to DNA of CAM, following various treatments, was expressed as mean  $\pm$  SEM and the results between various groups compared by the Kruskal-Wallis test. Coefficient of variation, CV,  $(\text{SD} \times 100)/\bar{x}$ , was also calculated.

#### Tissue extracts

Tumors or control tissues were homogenized in PBS (1 g/10 ml) for 3 min at  $4^{\circ}\text{C}$  and centrifuged to remove particulate material. Supernatants were decanted, dialyzed (4000 cut-off) against distilled water for 24 hr at  $4^{\circ}\text{C}$  then lyophilized and stored at  $-20^{\circ}\text{C}$  until use. All solutions of extracts in PBS were adjusted to the same (1%) protein concentration, applied onto CAM at a volume of 0.2 ml and incubated at  $37^{\circ}\text{C}$  for 22 hr followed by application of [ $^3$ H]thymidine for 2 hr.

#### EGF and drugs

Pure natural mouse epidermal growth factor (EGF) (synthesized by Dr. H. Gregory from I.C.I., Alderley Park, UK) was obtained from Prof. S. Konturek as a lyophilized sample. Doxorubicin and bleomycin were obtained from a local pharmacy. EGF and drugs were dissolved in phosphate buffered saline (PBS) just before use, applied onto CAM at a volume of 0.2 ml and incubated at  $37^{\circ}\text{C}$  for 22 hr followed by 2 hr incubation with thymidine.

## RESULTS

#### Visual assessment of angiogenesis

The angiogenic activities of various tissues are shown in Table 1. The maximal vascular response was attained on day 15 and occasionally on day 14. A typical spoke-wheel response to the implant of meningioma (scores: 4.0+) is shown in Figure 1. When slices of adenocarcinoma of endometrium (3 cases) were heated ( $100^{\circ}\text{C}$  for 30 min) they evoked angiogenic responses in CAM scored between 0.1-1.0+.

#### DNA content in CAM

Table 2 shows that DNA content per mg of CAM was similar in embryos of various ages. When the slices of normal endometrium were implanted on CAM, DNA content—measured on day 15—amounted to  $1.36 \pm 0.11$   $\mu\text{g}/\text{mg}$  of CAM (mean  $\pm$  SEM,  $n=6$ ) and was comparable to the DNA content of untreated CAM (Table 2).

TABLE 1. Angiogenic activities of various tissues on CAM

Tissue	Number of patients	Scores of activity					P
		0	0.1-1.0	1-2.0	2.1-3.0	3.1-4.0	
Control*	9	3	4	0	0	0	—
Carcinoma of endometrium*	12	0	1	3	7(2)	1(1)	<0.01
Meningioma	1	0	0	0	0	1	—

\*Normal endometrium.

Three specimens were separated from CAM by Millipore filter and corresponding scores are given in parentheses.



FIG. 1. CAM of 11-day old chick embryo incubated for 3 days with implanted meninges. The blood vessels show radial orientation forming a typical spoke-wheel.

TABLE 2. DNA content in CAM of embryos of various ages

Age of embryo	Tissue	DNA, $\mu\text{g}/\text{mg}$ of CAM (mean $\pm$ SEM)	Number of embryos
9	—	$1.40 \pm 0.12$	5
10	—	$1.24 \pm 0.46$	4
11	—	$1.18 \pm 0.08$	11
12	—	$1.44 \pm 0.20$	5
13	—	$1.20 \pm 0.07$	16

into DNA of CAM (Table 3). CVs of these measurements were between 13.1-17.2%.

Similar to tumor extracts, epidermal growth factor (EGF) stimulated incorporation of thymidine into DNA of CAM (Table 4), while cytostatics, doxorubicin and bleomycin inhibited incorporation of thymidine into DNA of CAM (Table 4). In the case of bleomycin some embryos died following drug treatment.

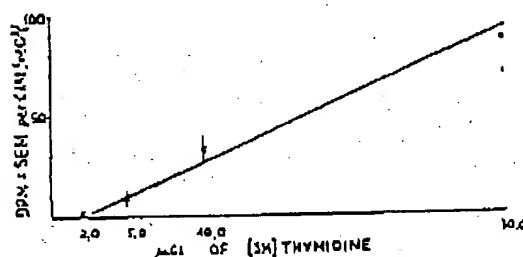
### DNA synthesis

Increasing amounts of  $[^3\text{H}]$ thymidine applied to CAM for 30 min produced linear incorporation into DNA (Fig. 2). Figure 3 illustrates the incorporation of thymidine to DNA when a constant dose ( $2.0 \mu\text{Ci}$ ) was applied onto CAM for various time intervals. Initial linear increase of incorporation of thymidine declined after 2.0 hr to a plateau. Coefficients of variation of most measurements were between 10-20%, and 2.0 hr time of incubation of  $[^3\text{H}]$ thymidine with CAM was chosen for further experiments. Experiments relating age of CAM with the degree of thymidine incorporation into DNA (Fig. 4) indicated that growth of CAM was largely completed by day 11 of embryo development. For subsequent experiments, 11 or 12-day old embryos were used.

Tumor extracts, when incubated with CAM for 24 hr, significantly increased incorporation of thymidine

### DISCUSSION

The visual assessment of angiogenic response of CAM of chick embryo precluded large-scale screening of potential angiogenic substances in this assay. A simple quantitative method introduced by Thompson *et*

FIG. 2. Effect of increasing concentrations of  $[^3\text{H}]$ thymidine applied to CAM for 30 min;  $n = 10$  for each dose. Mean  $\pm$  SEM are shown.

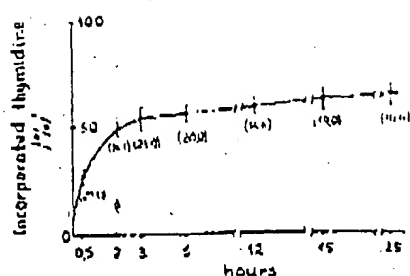


FIG. 3. Effect of constant dose (2.0  $\mu$ Ci) of [ $^3$ H]thymidine applied to CAM for various incubation times;  $n = 6$  for each time. Means  $\pm$  SEM are shown, coefficients of variation in parentheses. Open circles: experiments performed on eggs kept for 12 hr at room temperature.

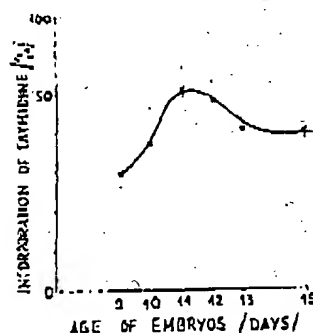


FIG. 4. Thymidine incorporation in CAM of various ages. CAMs were incubated with 2.0  $\mu$ Ci of [ $^3$ H]thymidine for 2 hr;  $n =$  minimum 4 for each day. Means  $\pm$  SEM are shown.

*et al.* (27) requires, because of low precision, large amounts of CAMs (see Introduction). The aim of the present work was such a modification of the procedure of Thompson *et al.* (27) which would increase the precision and allow for assay of individual eggs.

Our assay measured the ratio of thymidine incorporated into DNA to all forms of thymidine binding in CAM represented by the total radioactivity in the initial homogenate. Accordingly, the results were independent of the CAM's weight, and transfer of total radioactivity added to CAM *in vivo* was not critical. The DNA content per CAM's weight was similar during 10-15 days of embryo development (Table 2), also in the presence of the implant. We think that low precision of the original method (27) resulted from variation in the CAM's weight and uncontrolled losses of radioactivity during isolation of CAM. However, in the present assay, isolation of DNA required lipids removal and separation from tissue proteins. The simple method of isolation of DNA (23) appeared to be sufficient for the present purposes, as evidenced by the linear incorporation of thymidine to DNA (Fig. 2). In this experiment results were expressed as dpm per CAM, being the original data and were in agreement with that of Thompson *et al.* (27). The steady-state conditions for percent of thymidine incorporated to DNA were obtained after 2-3 hr of [ $^3$ H]thymidine incubation with CAM (Fig. 3) and 2 hr incubation time was chosen for present experiments. Figure 3 also shows that with our approach, precision of the assay was increased, as compared to the work of Thompson *et al.* (27), and coeffi-

TABLE 3. Effect of tumor extracts on [ $^3$ H]thymidine incorporation to DNA

Type of extract	Number of eggs	% of thymidine incorporated to DNA (mean $\pm$ SEM)
Phosphate buffered saline	7	59.8 $\pm$ 3.1
Normal endometrium (patient A.P.)	3	50.0 $\pm$ 4.0
Normal endometrium (patient G.K.)	9	50.8 $\pm$ 3.2
Adenocarcinoma endometrium (patient J.S.)	10	76.0 $\pm$ 1.0**
Adenocarcinoma endometrium (patient E.H.)	6	74.1 $\pm$ 5.4**
Adenocarcinoma endometrium (patient A.S.)	7	61.0 $\pm$ 1.4*
Meningioma (patient S.W.)	7	75.0 $\pm$ 3.0*

\* $p < 0.05$ ; \*\* $p < 0.01$  in comparison to parallelly run control.

\* $p < 0.01$  in comparison to saline.



TABLE 3. Effects of EGF, dexamethasone, and bleomycin on [<sup>3</sup>H]thymidine incorporation to DNA of CAM

Treatment	Number of eggs	% of thymidine incorporated to DNA (mean $\pm$ SEM)
EGF	9	35.7 $\pm$ 3.3
EGF + 0.2 $\mu$ g/egg	7	31.6 $\pm$ 3.3
EGF + 0.8 $\mu$ g/egg	4	60.4 $\pm$ 4.8
Dexamethasone (0.1 mg/egg twice at 0 and 12 hr)	5	12.1 $\pm$ 5.6**
Bleomycin (0.2 mg/egg twice at 0 and 12 hr)	3	18.0 $\pm$ 1.4*

\* $p < 0.05$ ; \*\* $p < 0.01$ 

of variation of the measurements throughout the entire study only exceptionally exceeded 20%. The rate of DNA synthesis was dependent on the age of the embryo. By day 11 of embryo development DNA synthesis was maximal (Fig. 3), in agreement with data of other reports (3, 27). Therefore, 11-12 day-old embryos were used for the present experiments.

The following data may indicate that the simple method of Montippon *et al.* (27) in our modification can quantitatively measure stimulation of angiogenesis on CAM. It has been demonstrated that meningioma, one of the most highly vascularized tumors, induced strong angiogenic response on CAM (20). We have confirmed this finding in the present work (Table 1, Fig. 1). Extract of meningioma was found to increase significantly ( $p < 0.01$ ) incorporation of thymidine to DNA as measured by our method (Table 3). This result is in agreement with autoradiographic studies of Kelly *et al.* (19), who observed the largest increase in thymidine labeling index following incubation of meningioma-conditioned medium with endothelial cultures. The other tumors which induced positive response on CAM assessed qualitatively, i.e., implants of adenocarcinoma of endometrium (Table 1), also significantly increased thymidine incorporation to DNA as assessed by our method (Table 3). The stimulating effects of tumor extracts was shared by EGF (Table 4), similar to its effect on vessels in the hamster chick pouch (24) and on endothelial cultures (9). In addition, dexamethasone and bleomycin, the DNA-synthesis inhibitors, significantly decreased incorporation of thymidine to DNA of CAM as evaluated by our assay (Table 4). This indirect evidence suggests that our method offers the possibility of quantitative assessment of angiogenesis on CAM.

In these studies we have also demonstrated that carcinoma of endometrium of the corpus uteri possesses angiogenic activity. This is, we believe, the first such observation. How angiogenic activity contributes to the spread of carcinoma of endometrium is not known. However, morphological studies are in progress to find out whether vessels of the uterus affected by the adenocarcinoma show signs of activity of angiogenic factor.

In the present method incorporation of thymidine in all cell types in CAM was followed. Therefore, the action directed specifically towards one type of cell would not be differentiated. This is the case of anti-angiogenic agents, protamine or heparin-cortisone mixture, specifically affecting endothelial cells, as opposed to cytostatics, acting on DNA of all cell types (26).

In contrast to anti-angiogenesis, stimulation of angiogenesis appears to involve all embryonic cells of CAM. All cell types in CAM are stimulated by various angiogenic factors, as evidenced by autoradiography (9, 27). Several growth factors such as epidermal and fibroblast growth factors (EGF and FGF) stimulate both angiogenesis *in vivo* and endothelial growth in culture (9, 24). Factors possessing intrinsic mitogenic activity, such as EGF, FGF, transforming growth factor- $\alpha$  (TGF- $\alpha$ ), tumor angiogenesis factor (TAF), and chondrosarcoma-derived endothelial cell growth factor are not cell-type specific (14, 16, 25, 29). It seems, therefore, that the present method is suitable for measurement of angiogenic activity of various tissues. Such a method should be useful for further studies on angiogenic factors.

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COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

IN THE MATTER OF: Australian

Patent Application 696764

(73941/94). In the name of:

Human Genome Sciences Inc.

- and -

IN THE MATTER OF: Opposition

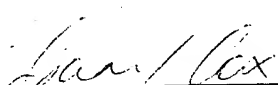
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Annexure GBC-19

This is **Annexure GBC-19** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
\_\_\_\_\_  
**Gary Baxter Cox**

WITNESS:

  
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Patent Attorney

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# Insulin delivery by somatic cell gene therapy

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## ABSTRACT

The feasibility of somatic cell gene therapy as a method of insulin delivery has been studied in mice. Murine pituitary ArT20 cells were transfected with a human proinsulin DNA in a plasmid containing a metallothionein promoter and a gene conferring resistance to the antibiotic G418. The ArT20MtIns-1.4 clone of cells was selected because of its higher insulin-releasing activity compared with other clones. After culturing for 24 h in Dulbecco's medium containing 10 mM glucose, the ArT20MtIns-1.4 cells released human insulin at about 5 ng/10<sup>6</sup> cells per 24 h. Insulin release was not significantly altered by raised concentrations of glucose, potassium or calcium, but insulin release was increased by 20 mM arginine, 5 mM isomethylbutylxanthine and 90 µM zinc.

ArT20MtIns-1.4 cells (2 × 10<sup>6</sup>) were implanted intraperitoneally into non-diabetic athymic nude

(nu/nu) mice, and the mice were made diabetic by injection of streptozotocin after 7 days. Release of human insulin *in vivo* was assessed using a specific plasma human C-peptide assay. Human C-peptide concentrations were maintained at about 0.1 pmol/ml throughout the 29 days of the study. The development of streptozotocin-induced hyperglycaemia was delayed in recipients of the cells releasing human insulin, compared with a control group receiving an implant of non-transfected cells. At autopsy the implanted ArT20MtIns-1.4 cells in each recipient had formed a tumour-like aggregation, with an outer region of insulin-containing cells. The study suggests that somatic cell gene therapy offers a feasible approach to insulin delivery.

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## INTRODUCTION

Somatic cell gene therapy has been considered as a potential method for insulin delivery in insulin-dependent diabetes (Selden *et al.* 1987b; Docherty, 1991). The principle of this approach is to introduce and express a new copy of the proinsulin gene in a somatic cell type which does not express the endogenous gene. This could be achieved by removing cells from a diabetic donor, and transfecting them with the proinsulin gene linked to a promoter. Cells incorporating and expressing the gene can be identified using a selectable marker such as an antibiotic resistance gene. The engineered cells can be grown in culture and returned to the donor, taking appropriate precautions for containment.

Cultured fibroblasts, COS cells and pituitary ArT20 cells have been transfected with genes encoding rat and human insulins (Lomedico, 1982;

Laub & Rutter, 1983; Moore *et al.* 1983; Diano-Zito *et al.* 1986; Selden *et al.* 1987b; Gross *et al.* 1989; Kawakami *et al.* 1992; Taylor & Docherty, 1992). Since proinsulin exerts only weak insulin-like activity *in vivo* (<10% of the potency of insulin) (Revers *et al.* 1984), it is important that proinsulin is processed to insulin. In this respect, the ArT20 cells provide a particularly useful model because they possess the requisite endopeptidases for processing proinsulin to insulin (Moore *et al.* 1983; Taylor & Docherty, 1992).

The present study investigated the feasibility of somatic cell gene therapy using ArT20 cells transfected with the human proinsulin gene. The insulin-releasing activity of these cells has been assessed *in vitro* and *in vivo* after implantation into immunoincompetent nude mice. The mice were subsequently treated with streptozotocin (STZ) to kill endogenous insulin-secreting islet B-cells and induce a state of insulinopenic diabetes. Insulin

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released by the implant was distinguished from endogenous murine insulins using an antiserum specific for the human C-peptide, which is co-secreted with the human insulin.

## MATERIALS AND METHODS

### Chemicals and animals

Cell culture reagents were from Gibco-BRL (Paisley, Strathclyde, U.K.), STZ was from Sigma Chemical Co. (Poole, Dorset, U.K.), human C-peptide antiserum, tracer and standards were from Novo Nordisk Diagnostics (Cambridge, Cambs, U.K.), insulin antiserum GP6 was a gift from Dr D. F. Steiner (Howard Hughes Medical Institute, University of Chicago, Chicago, IL, U.S.A.), rat C-peptide 1 was a gift from Dr S. Hampton (University of Surrey, Guildford, Surrey, U.K.) and high-performance liquid chromatography (HPLC)-grade ethanol was from Fisons Scientific Equipment (Loughborough, Leics, U.K.).

Adult male athymic nude (nu/nu) mice weighing 20–25 g were obtained from Bantin and Kingman (Hull, Humberside, U.K.). Mice were maintained in an isolated environment with filtered air at 22 °C, with 12 h light per day (08.00–20.00 h) and a standard pellet diet (Mouse Breeding Diet 1; Heygate, Northampton, Northants, U.K.) and tap water available *ad libitum*.

### Transfected cells

The AtT20 murine pituitary corticotrophic cell line, transfected with the pMtNeolns recombinant plasmid, was established and maintained as described previously (Taylor & Docherty, 1992). The plasmid contains a full-length human preproinsulin cDNA (511 bp) driven by the mouse metallothionein-1 promoter and ending in SV40 splice and polyadenylation sequences (Fig. 1). The plasmid also contains genes conferring resistance to ampicillin and G418, to serve as selectable markers. The pMtNeol plasmid was kindly supplied by Dr K. Peden, NIH, Bethesda, MD, U.S.A. The transfected cells, previously termed AtT20pMtNeohPPI/1 (Stewart *et al.* 1992), have been renamed AtT20MtIns-1 and the clone used in this study was AtT20MtIns-1.4.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 µg streptomycin/ml, 100 units penicillin/ml and a final glucose concentration of 11 mM. In certain experiments, glucose-free medium was supplemented with glucose at different concentrations. Culture

conditions were 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

### Characterization of AtT20MtIns-1.4 cells *in vitro*

Expression of the human preproinsulin gene was examined after culture of the cells for 48 h in medium supplemented with 90 µM zinc chloride and 5 µM cadmium chloride to enhance expression of the metallothionein promoter (Taylor *et al.* 1991). The medium was replaced with serum-free DMEM and the cells were cultured for a further 4 h. The medium was then removed, concentrated on a SepPak C18 column (Millipore, Watford, Herts, U.K.), and analysed by reverse-phase HPLC using a C18 reverse-phase column. Fractions were neutralized, lyophilized and resuspended in phosphate-buffered saline. The fractions were assayed for insulin-like immunoreactivity using a broad-specificity antiserum (GP6) which reacts similarly with insulin, proinsulin and partially split proinsulin intermediates.

The effects of various agents on insulin release were examined using quadruplicate cultures of  $1.5 \times 10^5$  cells for 24 h in 2 ml medium containing 10 mM glucose. The medium was sampled to assay for insulin and replaced with medium containing 10 mM glucose, 16.7 mM glucose or 10 mM glucose plus one of 15 mM potassium chloride, 7.6 mM calcium chloride, 20 mM arginine hydrochloride, 5 mM isomethylbutylxanthine (IBMX) or 90 µM zinc sulphate. Cells were cultured for a further 24 h and the medium was sampled for insulin assay.

### Implantation study

Insulin-releasing AtT20MtIns-1.4 cells ( $2 \times 10^6$ ) were implanted intraperitoneally into non-diabetic nude (nu/nu) mice, and a similar number of non-transfected pituitary cells was administered to a separate group of nude mice to serve as a control. Seven days after implantation, STZ (200 mg/kg i.p. in citrate buffer, pH 4.5) was administered. The study was continued until day 29. Body weight and food and fluid intake were monitored, and plasma glucose and plasma human C-peptide concentrations were determined at intervals using blood collected at 10.00 h from the tail in the freely fed state. Additional blood samples were collected on day 6 at 30 min after an oral glucose challenge (2 g/kg in a 40% (w/v) solution), and on day 7 after a 24-h fast, immediately before STZ administration.

At the end of the study the implants were identified within the abdomen of most recipients.

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### Assays

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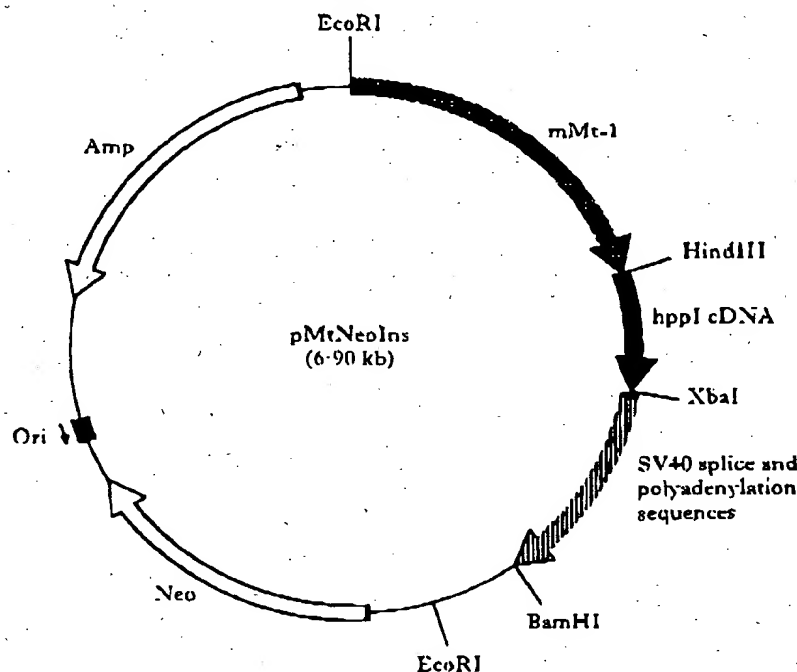


FIGURE 1. Plasmid map of pMtNeolns. The positions of the ampicillin resistance (Amp) and G418 resistance (Neo) genes, the mouse metallothionein promoter (mMt-1) and the SV40 splice and polyadenylation sequences are indicated. The human preproinsulin cDNA (hppI) was inserted between the metallothionein promoter and the SV40 sequences of pMtNeolns.

They were removed, weighed and subjected to routine histological examination with haematoxylin-eosin and aldehyde-fuchsin staining. In a separate study, the implants were extracted in 5 ml acid-ethanol/g (750 ml ethanol, 250 ml water, 15 ml concentrated hydrochloric acid) for human C-peptide assay.

#### Assays

Plasma glucose was determined by an automated glucose oxidase procedure (Stevens, 1971) and immunoreactive insulin-like material in HPLC fractions and cell culture media was measured by radioimmunoassay with polyethylene glycol precipitation (Shakur *et al.* 1989). Human C-peptide in plasma and implant extracts was measured by an ethanol-precipitation radioimmunoassay procedure (Heding & Rasmussen, 1975). The C-peptide antiserum (Novo K6) did not cross-react with rat C-peptide 1 and no reactivity was detected with plasma from normal fed rats and mice (assay sensitivity 0.01 pmol/ml). The inter- and intra-

assay coefficients of variation for standards (0.05–0.5 pmol/ml) were 11 and 2.7% respectively.

#### Statistical analysis

Groups of data are presented as means  $\pm$  s.e.m. Data were compared using Student's *t*-test. Differences were considered to be significant if  $P < 0.05$ .

## RESULTS

### Characterization of insulin gene expression

Expression of the human preproinsulin gene by AtT20Mtns-1.4 cells was associated mainly with the release of insulin (80%), together with the release of small amounts of proinsulin and partly split proinsulin intermediates (Fig. 2).

### Regulation of insulin gene expression *in vitro*

Under control conditions (10 mM glucose), the release of insulin-like immunoreactive material by

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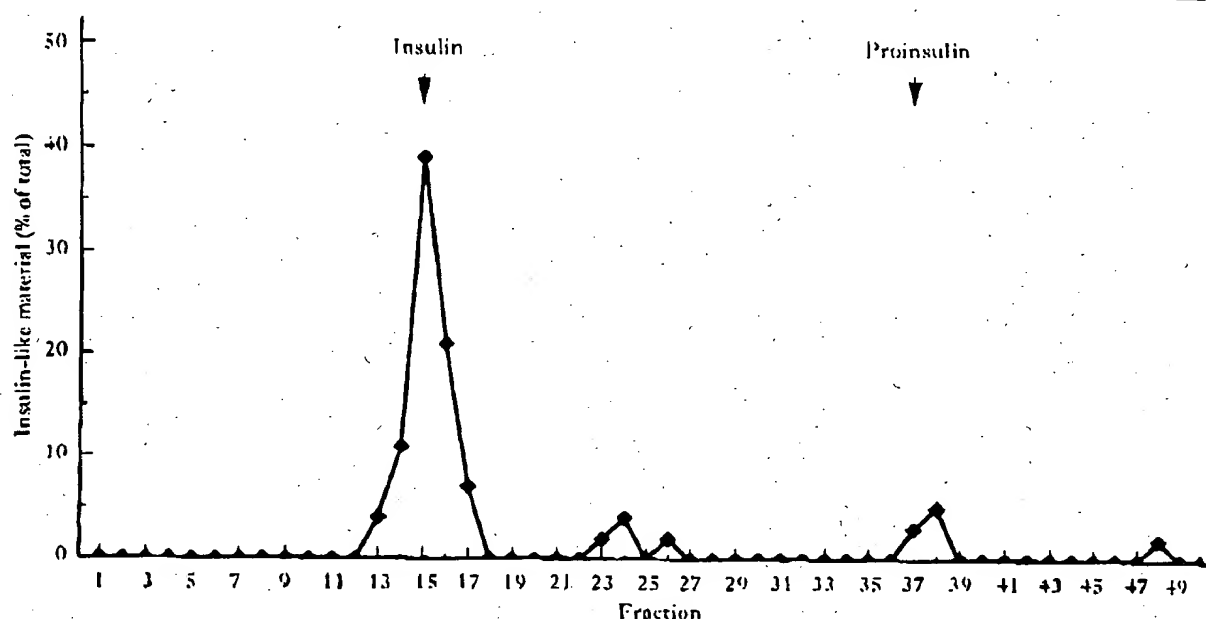


FIGURE 2. High-performance liquid chromatography elution profile of the insulin-like immunoreactivity released by the AtT20MtIns-1-4 cell line. Medium was removed from cultured cells as described in the Materials and Methods, concentrated on a SepPak C18 column, and analysed using a C18 reverse-phase column. Fractions were assayed for insulin-like immunoreactivity using a broad-specificity antiserum.

AtT20MtIns-1-4 cells was  $5.06 \pm 0.22$  ng/ $10^6$  cells per 24 h ( $n=24$ ). Insulin release was not significantly altered by incubation for 24 h in medium containing 16.7 mM glucose, 15 mM potassium chloride or 7.6 mM calcium chloride (Fig. 3). However, insulin release was increased by incubation in medium containing 20 mM arginine hydrochloride (275% increase), 5 mM IBMX (137% increase) or 90  $\mu$ M zinc sulphate (84% increase).

#### Implantation of AtT20MtIns-1-4 cells

Human C-peptide was not detected in the plasma of nude mice prior to implantation of the cells releasing human insulin, and was not detected after implantation of the control (non-transfected pituitary) cells (Fig. 4). However, 6 h after the intraperitoneal implantation of  $2 \times 10^6$  AtT20MtIns-1-4 cells, plasma human C-peptide was detected at 0.1 pmol/ml, and increased to a maximum of 0.16 pmol/ml by day 10 (Fig. 4a). Plasma human C-peptide concentrations of about 0.1 pmol/ml were maintained until the end of the study (day 29). The human C-peptide concentration was not significantly

altered at 30 min after an oral glucose challenge on day 6 ( $0.098 \pm 0.006$  pmol/ml,  $n=10$ ) or by a 24-h fast on day 7 ( $0.108 \pm 0.016$  pmol/ml,  $n=10$ ). Moreover, there was no change in plasma human C-peptide concentrations during the onset of STZ-induced diabetes. The human C-peptide concentrations in the mouse plasma were about one-quarter of the values in normally fed non-diabetic human subjects (mean value 0.4 pmol/ml) measured in the same assay.

In the non-diabetic state, plasma glucose concentrations were slightly reduced by the implantation of the AtT20MtIns-1-4 cells compared with the control pituitary cells (Fig. 4b). The hyperglycaemic response 30 min after an oral glucose challenge on day 6 was similar in the two groups ( $14.0 \pm 0.7$  mM,  $n=10$ , and  $14.8 \pm 0.8$  mM,  $n=11$ , in test and control mice respectively).

The high dosage of STZ (200 mg/kg i.p.) administered on day 7 produced a rapid and marked hyperglycaemia in controls, resulting in three fatalities by day 14 and subsequent termination of this group. In contrast, mice carrying the human insulin-releasing AtT20MtIns-1-4 cells showed a gradual rise in plasma glucose, with the development of severe hyperglycaemia being delayed by

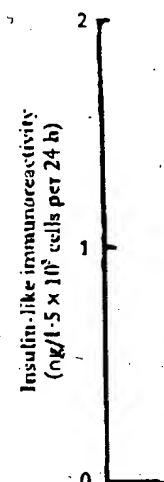


FIGURE 3. Insulin-like immunoreactivity released by AtT20MtIns-1-4 cells after 24 h in 2 mM glucose. Control medium yielded an ILI of 0.1 ng/1.5  $\times 10^6$  cells per 24 h,  $n=24$ . \* $P < 0.05$  vs. control. Values from test versus control.

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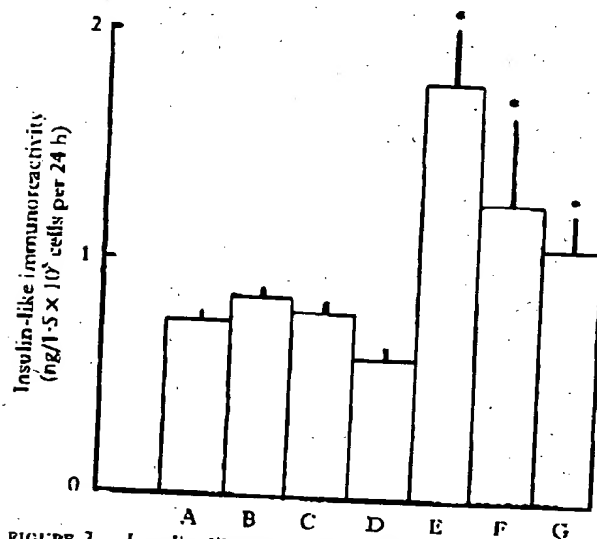


FIGURE 3. Insulin-like immunoreactivity (ILI) in the medium after culturing  $1.5 \times 10^5$  ArT20MrIns-1.4 cells for 24 h in 2 ml medium containing different agents. Control medium contained 10 mM glucose (A) and yielded an ILI value of  $0.76 \pm 0.03$  ng/ $1.5 \times 10^5$  cells per 24 h,  $n=24$ . Test media contained 16.7 mM glucose (B) or 10 mM glucose plus one of 15 mM KCl (C), 7.6 mM  $\text{CaCl}_2$  (D), 20 mM arginine hydrochloride (E), 5 mM isomethylbutylxanthine (F) or 90  $\mu\text{M}$   $\text{ZnSO}_4$  (G). ILI values from test media are means  $\pm$  S.E.M.,  $n=4$ , \* $P<0.05$  versus control (10 mM glucose) (Student's *t*-test).

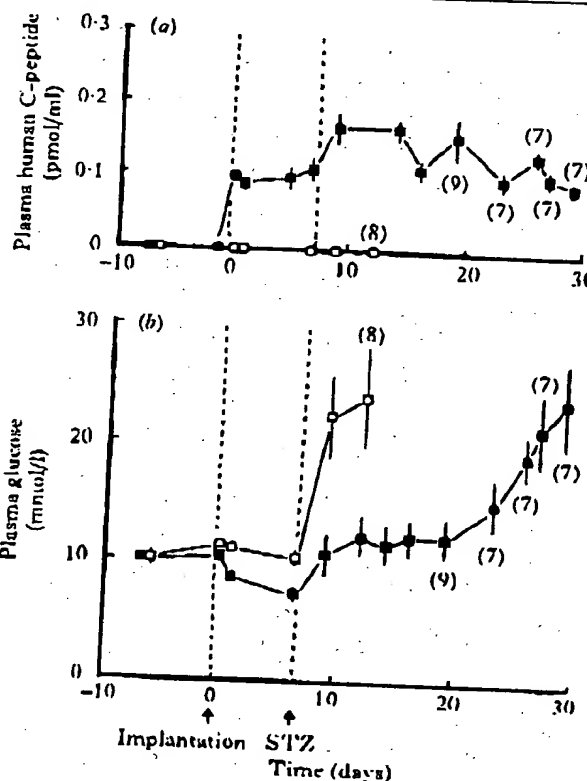


FIGURE 4. Implantation study: (a) plasma human C-peptide and (b) plasma glucose concentrations of nude (nu/nu) mice implanted intraperitoneally on day 0 with  $2 \times 10^6$  ArT20MrIns-1.4 cells (■) or non-transfected pituitary cells (□). Streptozotocin (STZ; 200 mg/kg i.p.) was administered on day 7. Values for the group implanted with non-transfected pituitary cells have been terminated at day 14 due to the high incidence of fatalities. Values are means  $\pm$  S.E.M. of 10 test and 11 control mice, except where shown by values in parentheses.

about 14 days. There was only one fatality by day 19 and two further fatalities up to day 29. Thus it appears that the release of insulin by implanted ArT20MrIns-1.4 cells was sufficient to defer the development of diabetes during the initial period of endogenous B-cell destruction by STZ. However, the ArT20MrIns-1.4 implant could not compensate for the hyperglycaemic effect of prolonged insufficiency of the endogenous B-cell population. It is noteworthy that the STZ diabetic nude mice became particularly insulin resistant, as indicated by only a slight hypoglycaemic response to subcutaneous administration of 10 U short-acting human insulin/kg (C. Stewart, unpublished observations). This is comparable with the failure of the insulin-releasing implant to prevent the eventual development of severe hyperglycaemia in STZ-treated nude mice.

Hyperphagia, polydipsia and body weight loss were observed after the induction of STZ diabetes in each group of mice. The extent of these features was consistent with the extent of hyperglycaemia (data not shown).

At the end of the study autopsies were performed on the mice. The ArT20MrIns-1.4 cells were identified in five out of seven recipients as a tumour-like aggregation (weighing about 50 mg) within the abdomen, adhering to the ventral peritoneum. Gross morphology of the cell aggregates revealed vascularization at the periphery with necrosis towards the centre. Peripheral cells stained positively for aldehyde-fuchsin.

In a separate study 30 days after implantation of  $2 \times 10^6$  ArT20MrIns-1.4 cells, the abdominal aggregations weighed  $56 \pm 13$  mg,  $n=5$ . The human C-peptide content of these aggregations was  $30.5 \pm 1.0$  pmol/g,  $n=5$ , corresponding to  $1.72 \pm 0.46$  pmol/aggregation.



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## DISCUSSION

The present study has shown that pituitary AtT20 cells can be stably transformed to produce and release human insulin after intraperitoneal implantation into athymic nude mice. Use of a specific assay for plasma human C-peptide has allowed the insulin-releasing activity of the implant to be monitored separately from that of endogenous islet B-cells. The release of human insulin by implanted cells was associated with a delay in the development of STZ diabetes.

Previous studies have inserted and expressed cDNA encoding human preproinsulin in fibroblasts (Moore *et al.* 1983; Diatloff-Zito *et al.* 1986; Selden *et al.* 1987b; Kawakami *et al.* 1992) and pituitary AtT20 cells (Moore *et al.* 1983; Taylor & Docherty, 1992). Unlike fibroblasts, which release proinsulin almost exclusively, AtT20 cells can process proinsulin to insulin. In AtT20 cells the insulin is co-localized and co-secreted with adrenocorticotrophic hormone (ACTH) via constitutive and regulated pathways (Moore *et al.* 1983; Orci *et al.* 1987; Powell *et al.* 1988), although the amount of insulin produced is much less than that of ACTH (Moore *et al.* 1983). The release of C-peptide (Moore *et al.* 1983; Powell *et al.* 1988) has been confirmed in the present study.

Regulation of the exogenous preproinsulin gene has been achieved *in vitro* with cyclic AMP (cAMP) analogues (Moore *et al.* 1983; Powell *et al.* 1988; Gross *et al.* 1989), and incubation with IBMX increased insulin release in the present study. It is possible that cAMP activates transcription factors which interact with the metallothionein promoter. Sensitivity of this promoter to zinc is well established (Hammer, 1986; Dickerson *et al.* 1989; Taylor & Docherty, 1992), and the exploitation of this mechanism to regulate cells transfected with the human growth hormone gene has been described (Selden *et al.* 1987a). Arginine strongly enhanced the expression of the preproinsulin gene in the present study, but the mechanism of this is unknown.

Attempts to deliver insulin by somatic cell gene therapy have previously been limited to the production of proinsulin from fibroblasts (Selden *et al.* 1987b; Kawakami *et al.* 1992). These studies have not distinguished the secretory products of the implanted cells from the endogenous production of insulin-like immunoreactive materials. This has been overcome herein with a specific human C-peptide radioimmunoassay, and with HPLC analysis of the secretory products *in vitro*.

When  $2 \times 10^6$  insulin-releasing AtT20MtIns-1.4 cells were implanted into non-diabetic mice, which

were subsequently made STZ diabetic, the implant continued to release insulin throughout the 29 days of the study. Although the plasma C-peptide concentration achieved by the implant was only about one-quarter of the normal C-peptide concentration (Heding & Rasmussen, 1975), the development of hyperglycaemia was delayed by about 2 weeks. Since STZ diabetic nude mice become very insulin resistant, this could explain why the implants did not prevent the eventual progression to severe hyperglycaemia. Indeed, the release of ACTH and other pro-opiomelanocortin-derived peptides (e.g. opiates) with which insulin is co-processed and co-secreted by the AtT20 cells (Moore *et al.* 1983; Orci *et al.* 1987; Powell *et al.* 1988) could aggravate the hyperglycaemia both directly (Bailey & Flatt, 1987a,b) and via stimulation of the ACTH-adrenal axis (Lenzen & Bailey, 1984).

The aggregation of implanted cells into a typically tumour-like organization was consistently observed. Even in mice with very low plasma C-peptide concentrations it was possible to detect insulin-containing cells in the outer regions of the cell aggregation. As substantiated by the small amounts of C-peptide extracted from these aggregations, there was only limited intracellular storage of insulin (about 30 pmol/g).

The present study provides evidence for the feasibility of delivering insulin by somatic cell gene therapy. By selecting a cell model (pituitary AtT20 cells) with an active secretory mechanism and appropriate endopeptidase activity, it has been possible to produce and release insulin. AtT20 cells express the high  $K_m$  glucose-phosphorylating enzyme glucokinase. Thus it has recently been shown that AtT20 cells transfected with cDNA for preproinsulin and the GLUT-2 glucose transporter exhibit glucose-stimulated insulin biosynthesis and release (Hughes *et al.* 1992). To apply this technology to somatic cell gene therapy will require the use of a convenient source of cells for primary culture, such as fibroblasts. These will require extensive genetic manipulation to achieve regulatable insulin release before implantation back into the donor (Selden *et al.* 1987b; Docherty, 1991; Hughes *et al.* 1992). While this approach avoids the need for immunoisolation, it will be necessary to introduce safeguards to contain the growth of implanted cells (Kawakami *et al.* 1992).

## ACKNOWLEDGEMENTS

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COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

IN THE MATTER OF: Australian

Patent Application 696764

(73941/94). In the name of:

Human Genome Sciences Inc.

- and -

IN THE MATTER OF: Opposition

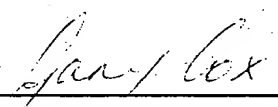
thereto by Ludwig Institute for Cancer

Research, under Section 59 of the

Patents Act.

Annexure GBC-20

This is **Annexure GBC-20** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
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**Gary Baxter Cox**

WITNESS:

  
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Patent Attorney

PEYTEE KHOO

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## MINIREVIEW

## Gene Therapy for Human Hemoglobinopathies (43665)

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The molecular defects in sickle cell disease and  $\beta$ -thalassemia have been well characterized and seem amenable to genetic correction (1). The development of effective genetic therapy could revolutionize treatment of the hemoglobinopathies. Before envisioning treating patients, methodologies will be required to ensure safe, efficient, and stable transfer of globin genes into hematopoietic stem cells and subsequent high-level gene expression in mature erythroid cells. This minireview will focus on the use of viral gene transfer vectors as potential therapeutic agents for the treatment of human hemoglobinopathies.

The thalassemias and clinically significant hemoglobinopathies are among the most common single gene disorders throughout the world. Patients with severe phenotypes rely on regular erythrocyte transfusions that can be associated with life-threatening iron overload despite intensive chelation (2). Long-term transfusion therapy may result in the development of anti-erythrocyte antibodies making subsequent transfusions difficult or, in some instances, impossible (3, 4). Allogeneic bone marrow transplantation has been performed with some success but is feasible in only a small percentage of affected patients (5, 6). Recent work has focused on the pharmacologic manipulation of fetal hemoglobin. Underpinning these efforts is the premise that increased  $\gamma$ -globin gene transcription and fetal

hemoglobin synthesis leads to more effective erythropoiesis and/or decreased hemolysis in patients with  $\beta$ -thalassemia and sickle cell disease (7-9). These treatments are, however, potentially toxic with unknown long-term complications.

## Globin Gene Organization

Hemoglobin is a tetrameric protein composed of two dimeric polypeptide units encoded by two different gene families on two separate chromosomes. The  $\alpha$ -globin gene cluster, located on chromosome 16, includes the duplicated  $\alpha$  genes ( $\alpha_1$ ,  $\alpha_2$ ) present in the fetal and adult stages of erythropoiesis and the embryonic  $\zeta$ -gene. Located on chromosome 11 are the cluster of  $\beta$ -like genes including the two adult genes,  $\delta$  and  $\beta$ , the two fetal genes,  $\gamma^A$  and  $\gamma^G$ , and the embryonic  $\epsilon$ -gene (see Fig. 1). During normally developing erythropoiesis, six distinct hemoglobin species are present in the transition from intrauterine to adult life. Coordinated gene expression in the  $\alpha$ - and  $\beta$ -gene clusters occurs at each site of erythropoiesis: the yolk sac of the embryo, liver of the fetus, and bone marrow postnatally. This process of coordinated expression, known as "hemoglobin switching," coincides with the change in hemoglobin phenotype. The  $\beta$ -like genes are activated and silenced in the 5' to 3' order of their transcriptional positions along chromosome 11.

Current switching models suggest competition between the individual  $\beta$ -like genes for regulatory elements defined within the distant DNase I hypersensitive sites known collectively as the locus control region (described below). A putative switching factor(s) is involved with either the silencing and/or activation of the  $\beta$ -gene cluster. Interaction of switching factors with  $\beta$ -like gene promoters may determine whether active gene transcription occurs. An example is the identification of a stage selector element in the human  $\gamma$ -

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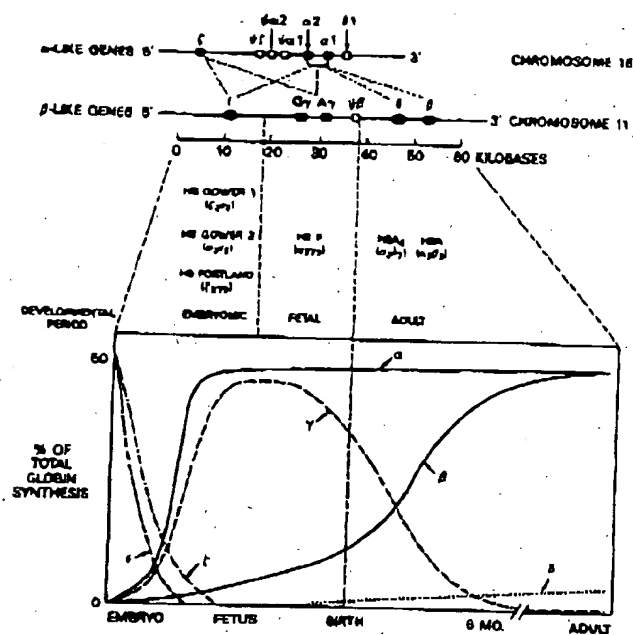


Figure 1. The spatial organization of the  $\alpha$ - and  $\beta$ -globin gene clusters (top). Coordinated globin gene expression and hemoglobin switching (center) at each stage of erythrocyte development (bottom) (adapted from Ref. 87).

globin gene promoter and of the nuclear protein which binds to this element and enables the  $\gamma$ -gene to competitively silence the  $\beta$ -globin gene (10). Levels of the specific DNA-binding protein are higher in more developmentally immature cells in which  $\gamma$ -globin expression is elevated. Analysis of the  $\alpha$ -like globin gene cluster suggests a similar type of regulation (11).

### Regulation of Globin Gene Transcription

The expression of the individual globin genes is regulated at the level of gene transcription, as supported by measurement of globin transcriptional rates and by quantitation of globin mRNA from patients with thalassemia (12). In general, globin transcriptional regulation requires cis-acting DNA sequences located within the globin gene cluster and trans-acting factors which bind sequence-specific motifs within the cis-acting regulatory elements.

Regulation of the human  $\beta$ -globin gene cluster ( $\epsilon$ ,  $\gamma^A$ ,  $\gamma^G$ ,  $\delta$ ,  $\beta$ ) is mediated via local cis-acting sequences including the globin promoters and enhancers 3' of the  $\gamma$ - and  $\beta$ -genes. Initial efforts to define cis-acting elements responsible for globin gene expression in transgenic animals revealed that local sequences were not sufficient for normal globin expression. The observation that deletions upstream of the  $\beta$ -globin gene inactivated globin expression suggested that other regulatory elements were necessary. These distant regulatory elements that flank the  $\beta$ -globin cluster are associated

with DNase I hypersensitive sites (HS), and inclusion of these sites modulates high level globin expression. These sites are collectively termed the locus control region (LCR). Four sites (5' HS1-4) are located several kilobases 5' to the  $\alpha$ -globin gene, and one site (3' HS1) is mapped 3' to the  $\beta$ -globin gene. The active elements of the LCR are encompassed within 300-400 base pairs of DNA found at each HS (13, 14). The HS2, 3, and 4, when linked to globin genes singly or in combination, substantially increase globin gene expression in transfected erythroleukemia cells or when introduced into transgenic animals (15).

Recently, several erythroid-specific enhancer sequences and trans-acting factors have been defined that appear to regulate globin gene transcription. One of the most powerful enhancer elements in the  $\beta$ -globin locus lies within the HS2 and is localized to tandem AP-1 binding sites (16). This element is required for high level  $\gamma$ -globin gene expression in stably transfected K562 cells. K562 cells provide a model for the study of globin gene regulation and have been used to define important cis-acting regulatory elements. The HS2 enhances by 150-fold transcriptional activity in hemin-induced K562 cells but is relatively inactive in nonerythroid cells. The trans-acting factor NF-E2 binds to the HS2 enhancer and is required for hemin-inducible activity of the enhancer (17). Transgenic animal experiments using only the HS2 site linked to a  $\beta$ -globin gene enabled the production of 25-50% levels of endogenous globin transcript (18). This factor has been characterized as a 45-kDa basic-leucine zipper DNA binding protein expressed in erythroid and megakaryocytic lineages (19).

The erythroid-specific transcription factor NFE-1 (GATA-1) binds to GATA consensus motifs found in several of the cis-acting elements. Experiments with transgenic mice indicate that GATA-1 may be important in the development and function of red blood cells (20, 21).

### Globin Pathophysiology

The thalassemic syndromes are hereditary anemias which occur due to mutations that affect the synthesis of either  $\alpha$ - or  $\beta$ -globin chains. The ratio of  $\alpha$ - to  $\beta$ -chain synthesis is the major determinant of pathology. Excess of either globin chain can lead to the formation of aggregates or intracellular inclusions causing decreased red blood cell membrane deformity, ineffective erythropoiesis, and accelerated red cell destruction. Discussion of clinically relevant severe thalassemia syndromes is usually directed toward the  $\beta$ -thalassemias.

$\beta$ -Thalassemia refers to inadequate  $\beta$ -globin chain synthesis encoded by a single  $\beta$ -globin gene on chromosome 11. Heterozygous individuals are characterized by a quantitative deficiency of  $\beta$ -globin production relative to  $\alpha$ -globin. In homozygous patients with  $\beta$ -

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thalassemia, deficient or absent  $\beta$ -globin gene synthesis causes the production of poorly hemoglobinized, defective erythrocytes resulting in hemolysis and severe anemia (22). Patients with severe disease require frequent red blood cell transfusions with attendant iron accumulation. The severity of this disease is modulated by increased  $\gamma$ -globin synthesis and increased fetal hemoglobin ( $\alpha_2\gamma_2$ , Hb F) or concomitant  $\alpha$ -thalassemia.

In homozygous sickle cell anemia, the mutant hemoglobin (Hb S,  $\alpha_2\beta_2^s$ ) is susceptible to polymerization resulting in altered erythrocyte rheological properties, vaso-occlusion, and multiorgan damage. The severity of sickle cell disease correlates with the degree of hemoglobin polymerization. Hb F has a sparing effect on polymerization and decreases the tendency of Hb S to precipitate within the erythroid cells.

In these disease states, the transfer and expression of either  $\beta$ - or  $\gamma$ -globin genes should be highly effective in correcting these genetic defects. Replacement with a functional  $\beta$ -globin gene could correct the defect in severe  $\beta$ -thalassemia, whereas gene insertion of a  $\gamma$ -globin gene could ameliorate the potential for polymerization in sickle cell disease. Studies of sickle cell and  $\beta$ -thalassemia patients reveal that mild or absent clinical manifestations occur in the presence of hemoglobin F levels of 20-40% (23, 24). Expression of a  $\beta$ -globin transgene at 10-20% of normal endogenous levels is adequate to achieve a dramatic phenotypic improvement in a thalassemic mouse model (25).

#### Gene Transfer

The following are the major requirements for globin gene transfer to be clinically applicable. (i) Transduced (introduced) globin gene expression must be at sufficient levels. (ii) Expression should be stably regulated and erythroid specific. (iii) The totipotent bone marrow stem cell should be transduced at high frequency and/or maintain a competitive advantage over nontransduced cells for its self-renewal. (iv) The introduction of foreign DNA into the target cell genome should have limited potential for insertional mutagenesis and/or endogenous gene disruption.

Ideally, replacement of a defective gene with the correct genetic sequence by targeting genes to specific sites within the genome would be an optimal method for genetic therapy. Homologous recombination into a  $\beta$ -globin locus has been achieved in cultured embryonic stem cells, albeit at a frequency too low to be of any current therapeutic value (26). However, as a result of recent advances in molecular biology, the introduction of new genetic material by nonhomologous recombination with gene insertion into a target cell genome now appears attainable.

Gene transduction is accomplished by a variety of techniques, including viral vectors, poly-lysine/DNA conjugates, and other physical techniques. Viral gene

transfer vectors make use of the inherent efficiency of viruses to transfer and express their genetic information in mammalian cells (for review, see Ref. 27). As now envisioned, hematopoietic stem cells from an affected patient could be infected with an appropriate viral vector containing a correctly functioning globin gene. We will describe current work with retrovirus and the parvovirus, adeno-associated virus, as potential gene transfer vectors.

#### Retroviral Vectors

Retroviruses contain a single-stranded RNA genome that, upon entry into a cell, is converted into a double-stranded DNA before its integration into the host cell chromosome. Early interest in these viruses stemmed from their ability to induce tumors by insertional mutagenesis. The integrated provirus, containing its own powerful transcriptional elements, is thought to activate nearby proto-oncogenes or inactivate tumor suppressor genes. To avoid the possibility of wild-type retroviral infection, packaging cell lines are used to produce replication-defective recombinant retroviral particles. Vector and packaging cell strategies have been well documented (27).

**Globin Retroviral Vectors.** Transfer of genomic human  $\beta$ -globin sequences using retroviral vectors has evolved with both the development of improved packaging cell lines and greater understanding of globin gene regulation. Initial experiments utilized a 3.0-kb fragment of genomic  $\beta$ -globin into an ecotropic vector in both orientations (28). A neomycin resistance gene in those constructs was employed to rapidly screen for high titer producer clones subsequently used to generate infectious recombinant retroviral virions. The marker also facilitated isolation of target cells transduced by the retrovirus. Only the reverse orientation construct was functional and sufficient for proviral integration and subsequent viral production. Individual clones contained a single transferred proviral copy. Human  $\beta$ -globin transcripts were detected and the level of expression increased in dimethyl sulfoxide-induced MEL clones. Dimethyl sulfoxide, hemin, and other agents have been used in several human and murine leukemic cell lines to induce  $\beta$ -globin cluster gene expression. Furthermore, negligible globin expression was detected in 3T3 fibroblasts, indicating that the construct carrying a  $\beta$ -globin promoter contributed to erythroid-specific expression. The level of expression (compared with endogenous murine  $\beta$ -globin expression) was approximately 0.01%. Similar experiments using an amphotropic vector containing a neomycin resistance gene with a  $\gamma$ - $\beta$  globin hybrid were used to infect MEL cells with human  $\beta$ -globin expression at 10% of the endogenous induced expression (29). In both instances, viral titer was low and the provirus rearranged in some of the clones tested. Constructs containing portions of the

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5' untranslated region and intron 2 in the reverse orientation interfered with generation of full-length transcripts and yielded low titer recombinant virus (30). The 5' region could be removed, but the intron 2 was required for expression. Reverse orientation globin constructs may contain polyA termination signals, possibly accounting for abbreviated transcription and low titer virus generation.

Examination of retrovirally transduced  $\beta$ -globin in human hematopoietic cells demonstrated gene transfer and expression in erythroid colonies (burst-forming unit, erythroid) (31). Expression reached 5% of the endogenous  $\beta$ -globin in several pooled colonies using RNase protection assay. Expression was determined by using a 6-base pair insertion ("marking") at the 5' untranslated region to allow detection of the transferred gene. Infection frequency was low (0.04%) and attributed to the low viral titer ( $5 \times 10^4$  colony-forming units/ml). A truncated  $\beta$ -globin minigene (lacking introns) was also tested in MEL cells but expressed at undetectable levels regardless of the orientation.

The first *in vivo* experiment described a recombinant retroviral construct encoding a human  $\beta$ -globin gene that was used to infect murine hematopoietic cells and reconstitute transplanted mice (32). Expression was limited primarily to the erythroid lineage and varied from 0.4% to 4.0% of the endogenous mouse  $\beta$ -globin mRNA level. The proviral copy number per cell ranged from 0.02 to 0.40 copies/cell, found in all lineages. Long-term human  $\beta$ -globin gene expression was detected in transplanted animals at 4-9 months. The infection rate was low (18 of 104 animals reconstituted with infected bone marrow), which indicated that the marrow infection conditions needed to be optimized (increased viral titer, enrichment of pluripotent cells, and induction of quiescent stem cell cycling).

Confirmatory experiments in several laboratories demonstrated  $\beta$ -globin retroviral transfer in murine hematopoietic cells (33, 34). Long-term expression in all lineages from secondary recipient animals indicated that pluripotent stem cells rather than committed progenitor cells were infected. Co-culturing conditions for bone marrow target cells with recombinant retrovirus improved, largely through the inclusion of hematopoietic growth factors to shorten  $G_0$  and promote entry into cell cycle required for retroviral replication and integration (35). Despite improved transduction frequency in pluripotent bone marrow cells, globin expression still ranged from 1% to 5% of the endogenous level.

The recently discovered LCR regulatory elements flanking the  $\beta$ -globin gene cluster suggested a new approach to the design of retroviral vectors (36). Individual LCR fragments were included within a marked  $\beta$ -globin/neomycin gene cassette and used to generate recombinant amphotropic virions infectious for MEL

cells (37). One construct incorporating an HS2 fragment resulted in high level expression in a few clones (three), but with extreme expression variability (10-310%). The viral titers were  $10^4$ - $10^5$  colony-forming units/ml. Subsequently, investigators from several laboratories have been unable to generate LCR-globin producer lines of sufficient titer that do not exhibit proviral rearrangement or deletion. A recent report describes the use of a 36-base pair sequence encompassing the NFE-2 binding site within the HS2 region linked to human  $\beta$ -globin (38). The level of  $\beta$ -globin expression increased marginally from 6.0% to 12.0% with the addition of the enhancer element. Viral titers were again low, and introduction of multiple copies of the 36-base pair fragment promoted gross proviral rearrangement.

Generation of an ecotropic retrovirus containing an LCR cassette with truncated HS 4, 3, 2, and 1 sites linked to a human  $\beta$ -globin yielded 60-70% expression in MEL cells compared with endogenous murine globin expression. Transfer into murine hematopoietic progenitors and subsequent transplantation into lethally irradiated recipients resulted in human  $\beta$ -globin expression. These experiments suggest that inclusion of LCR elements may support high level  $\beta$ -globin gene expression in murine hematopoietic stem cells; however, significant rearrangement of the provirus occurred and the vectors employed yield low recombinant viral titers (39).

Many of the alternative viral vectors currently available either do not integrate into host cells at high frequency, are not easily rescuable from the integrated state, are limited in their host range, or include other viral genes, thereby creating a need for the development of a safe and efficient viral vector system. We feel that the human DNA virus, adeno-associated virus, offers a promising alternative to the currently utilized vectors.

#### Adeno-Associated Virus

Adeno-associated virus (AAV) is a defective member of the parvovirus family. The AAV genome is encapsidated as a single-stranded DNA molecule of plus or minus polarity (40, 41). Strands of both polarities are packaged, but in separate virus particles (42) and both strands are infectious (43). The single-stranded DNA genome of the human virus AAV-2 is 4675 base pairs in length (44) and is flanked by inverted terminal repeated sequences of 145 base pairs each (45). The first 125 nucleotides form a palindromic sequence that can form a T-shaped hairpin structure and can exist in either of two orientations (designated flip or flop). This unique structure has led to the suggestion (46) that AAV may replicate according to a model first proposed by Cavalier-Smith (47) in which the terminal hairpin of AAV is used as a primer for the initiation of DNA replication. The AAV sequences that are required

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*in cis* for packaging, integration/rescue, and replication of viral DNA appear to be located within a 191-base pair (bp) sequence that includes the terminal repeat sequences (48, 49).

The viral DNA sequence displays two major open reading frames, one in the left half and the other in the right half of the conventional AAV map (43). At least three regions which, when mutated, give rise to phenotypically distinct viruses have been identified in the AAV genome (50). The rep region, which occupies the conventional left half of the genome, encodes one or more proteins that are required for DNA replication and for rescue from the recombinant plasmid. The cap and lip regions appear to encode for AAV capsid proteins; mutants within these regions are capable of DNA replication but do not produce virus (50). AAV contains three transcriptional promoters, p5, p19, and p40 (45, 51-53).

AAV-2 can be propagated as a lytic virus or maintained as a provirus, integrated into host cell DNA (54). In a lytic infection, efficient replication requires coinfection with either adenovirus (55, 56) or herpes simplex virus (57)—hence the classification of AAV as a "defective" virus. When no helper virus is available, AAV can persist in the host cell genomic DNA as an integrated provirus (58, 59). Virus integration appears to have no apparent effect on cell growth or morphology (60, 61). Studies of the physical structure of integrated AAV genomes (59, 62) suggest that viral insertion into the host chromosome is usually in a tandem head to tail orientation and occurs within the AAV terminal repeated sequence. Integrated AAV genomes are stable, persisting in tissue culture for greater than 100 passages (59). Although AAV is a human virus, its host range for lytic growth is unusually broad. Virtually every mammalian cell line evaluated (including a variety of human, simian, canine, bovine, and rodent cell lines) can be productively infected with AAV, provided that an appropriate helper virus is used (i.e., canine adenovirus in canine cells) (54). These same cells are also capable of establishing an AAV latent infection in the absence of helper.

Despite the wide range of susceptible cell types, no disease has been associated with AAV in either human or animal populations (63), even though exposure is commonplace. Anti-AAV antibodies have been found frequently in humans and monkeys. Estimates suggest that about 70-80% of infants acquire antibodies to AAV types 1, 2, and 3 within the first decade; more than 50% of adults have been found to maintain detectable anti-AAV antibodies. AAV has been isolated from fecal, ocular, and respiratory specimens during acute adenovirus infections, but not during other illnesses (64).

**Infectious AAV Clone.** We initially cloned intact duplex AAV DNA into the bacterial plasmid pBR322

(65) and found that the AAV genome could be rescued from the recombinant plasmid by transfection of the plasmid DNA into human cells with adenovirus 5 as helper. The efficiency of rescue from the plasmid was sufficiently high to produce yields of AAV DNA comparable to those observed after transfection with equal amounts of purified virion DNA. The AAV sequences in the recombinant plasmid could be modified, and then "shuttled" into eukaryotic cells by transfection. In the presence of helper adenovirus (Ad), the AAV genome was found to be rescued free of any plasmid DNA sequences and replicated to produce infectious AAV particles (65-68). This developed an approach for mutant construction (67) that enabled us and others to explore viral gene function (43, 69), and to identify the *cis*-acting sequences needed for AAV rescue, replication, packaging, and integration (49).

AAV has been tested as a viral vector system to express a variety of genes in eukaryotic cells. Hermonat and Muzyczka (69) produced a recombinant AAV (rAAV) viral stock in which the neomycin resistance gene (*neo*) was substituted for the AAV capsid region and observed rAAV transduction of neomycin resistance into murine and human cell lines. The stable integrated viral vector could be rescued to produce replicating rAAV sequences after superinfection with Ad and wild-type AAV. Tratschin *et al.* (70) created an rAAV that was found to express the chloramphenicol acetyltransferase gene in human cells under the AAV p40 promoter. LaFace *et al.* (71) observed gene transfer into hematopoietic progenitor cells using an AAV vector. Wondisford *et al.* (72) cotransfected cells with two different recombinant AAV vectors, each encoding a subunit of human thyrotropin, and observed expression of biologically active thyrotropin.

The desirable size of inserted non-AAV or foreign DNA is limited to that which permits packaging of the rAAV vector into virions, and this depends on the size of retained AAV sequences. Tratschin *et al.* (70) constructed an AAV/chloramphenicol acetyltransferase genome that was 3% (approximately 150 nucleotides) longer than the wild-type AAV genome, and found that it could be packaged into virions. An AAV genome too large to be packaged resulted from insertion of a 1.1-kbp fragment of bacteriophage- $\lambda$  into a nonessential region of AAV (R. J. Samulski and T. Shenk, unpublished). Thus, the total size of the rAAV to be packaged into virions should be about 4800-5000 nucleotides in length.

As mentioned above, several AAV vector systems have been designed that contain a recombinant plasmid capable of being packaged into AAV particles. The recombinant virus generated then functions as a vector for stable maintenance or expression of a gene or a DNA sequence in eukaryotic cells when under control of an AAV or SV40 transcriptional promoter. However,



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a common problem encountered in all these AAV vector systems has been the inability to produce recombinant virus stocks free of helper AAV virus. Various methods have been used in attempts to decrease the percentage of contaminating helper virus (73). This problem has been a major drawback in the use of AAV as a prevalent viral vector. Our recent work, however, has succeeded in generating high titer viral stocks that are free of helper virus.

**AAV Vectors.** We have recently developed a method for producing substantially helper-free stocks of rAAV that can be used to efficiently and stably transduce foreign genes into host cells or organisms (49). Our present method for producing recombinant stocks is directed toward producing a viral expression vector system with improved efficiency, applicability, definition, and safety relative to viral vector systems currently utilized. The method utilizes a two-component system comprised of functionally, but not structurally, related rAAV genomes, one of which contains a segment of foreign DNA (the vector) but lacks DNA sequences necessary for viral replication, and the other (the helper AAV) which provides those viral functions not encoded by the vector but which cannot itself be incorporated into virions. Importantly, the vector and the helper DNA are sufficiently nonhomologous so as to preclude homologous recombination events that could generate wild-type AAV. Along with this development of the vector, we have conducted a study characterizing natural AAV integration. In this study, we have encountered the unexpected observation that wild-type AAV utilizes site-specific integration when establishing viral latency (see below).

**Production of the AAV Vector System.** We have constructed an infectious adeno-associated viral genome that contains two *Xba*I cleavage sites flanking the viral coding domain (43) (Fig. 2); these restriction enzyme cleavage sites were created to allow nonviral sequences to be inserted between the cis-acting terminal repeats of AAV (49). The AAV helper plasmid termed pAAV/Ad contains adenovirus type 5 terminal sequences (107 bp) in place of the normal AAV termini. This helper cannot be packaged into AAV virions, since it lacks the terminal cis-acting domain required for this function. The AAV terminal sequences were originally substituted with adenovirus terminal sequences in pAAV/Ad so as to transcriptionally enhance AAV gene expression (74). This hybrid plasmid did not contain the Ad packaging sequences (75) and therefore could not be packaged into Ad virions either.

The presence of the adenovirus termini substantially enhanced the expression of AAV-specific proteins from pAAV/Ad DNA when compared with pAAV/no TR (DNA which contained neither adenovirus nor AAV terminal sequences). When the helper plasmid pAAV/Ad and a vector pAAV/NEO were co-trans-

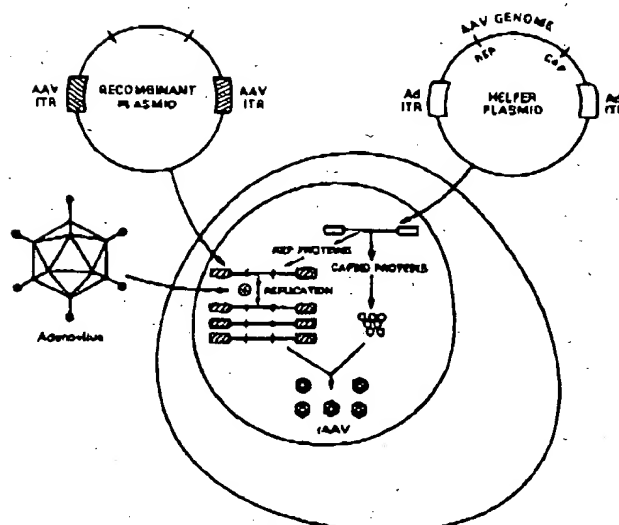


Figure 2. Generation of helper-free recombinant adeno-associated virions. A producer cell line is co-transfected with "helper" and recombinant (rAAV) plasmids. Helper plasmid generates rep and cap protein synthesis required for rAAV replication and encapsidation. Adenovirus infection provides necessary replication and packaging functions (see text for details).

ected into human cells in the presence of adenovirus, rescue and replication of the AAV/NEO sequences were detected. The vector viruses generated from this complementation contain only the terminal 191 nucleotides of the viral chromosome, demonstrating that all cis-acting AAV functions required for replication and virion production are located within that region. Recombinant viral DNA carrying a drug resistance marker gene were integrated into the cellular genome. These transduced genes could not be excised and replicated when the cells were subsequently infected with adenovirus, suggesting another level of safety (49). Thus, the AAV termini (145 bp) are sufficient for integration of the AAV chromosome into a host cell genome. No AAV gene expression is required to establish a latent infection using this vector, and up to 70% of the cells can be transduced.

**Site-Specific Integration.** The proviral integration form of wild-type AAV is a unique feature of this virus. Initial studies characterizing the AAV proviral state using restriction digestion and Southern blotting of genomic DNA demonstrated that the proviral DNA was covalently linked to cellular DNA in tandem concatamers (59, 76). These results have been confirmed and extended by the development of a protein-DNA binding enrichment technique used to isolate AAV proviral DNA from latent human cell lines (77). The strategy for retrieving AAV-cellular junctions involved a protein filter binding procedure that relied upon the interaction between  $\lambda$  repressor and its operator sequences. An infectious recombinant clone was used to

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generate an AAV- $\lambda$  hybrid that contained the  $\lambda$  operator site. The recombinant virus was used to establish latently infected AAV- $\lambda$  cell lines. Genomic DNA isolated from the latent cell lines digested with restriction enzymes were mixed with purified  $\lambda$  repressor protein and passed over a membrane filter. Southern hybridization analysis of the retained fragments showed a physical linkage between AAV proviral DNA and cellular sequences. Nucleotide comparison of clonal cellular sequences demonstrated viral-cellular junction rearrangements involving deletion of portions of the terminal repeats. An unrearranged preintegration junction cellular sequence used as probe confirmed the sequence location at chromosome 19. Polymerase chain reaction amplification using AAV and junction-specific primers generated viral/junction breakpoints that lay within a 100-bp sequence on chromosome 19. *In situ* analysis of latently infected cell chromosomes using AAV-specific probes further demonstrated that viral DNA integrated into only one locus. Both single and multiple copy number insertion patterns were located within this integration region.

The minimal elements required for AAV integration are currently being delineated. AAV vectors containing only the inverted terminal repeat integrate at high frequency, suggesting the importance of the inverted terminal repeat for integration. This also indicates that AAV integration relies on host cellular enzymes. Furthermore, work in our laboratory demonstrated the lack of site-specific integration with recombinant vectors containing only AAV termini (78). Taken together, this would imply that viral *trans*-acting factors are required for site-specific integration. Since limited amplification of the AAV genome is required for integration, the AAV *rep* proteins described previously are likely candidates for this function.

Since this initial observation, we have documented site-specific integration in a number of cell types (human T cells, colon, lung, and monkey kidney cells). Our preliminary characterization of this chromosomal region has revealed that (i) this sequence is highly conserved in primates, (ii) this chromosomal sequence appears not to be expressed in either latent or non-latent HeLa cells, and (iii) the integration site maps to the Q arm of chromosome 19. Extensive characterization of this chromosomal region will be essential in understanding regulation of rAAV transduced genes. For this reason, we have recently isolated two overlapping cosmid clones that hybridize to the chromosome 19 integration sequence (N. Epstein and R. J. Samulski, unpublished results). Further analysis of this region should be illuminating regarding both the integration mechanism and the potential of targeted gene delivery.

**AAV Vectors Expressing Globin Genes.** During our characterization of AAV vectors for targeted integration, we initiated a study to test this minimum AAV

vector for the efficient transduction and expression of globin gene sequences in the erythroid cell line K562. We constructed an rAAV vector containing the human  $\gamma^A$ -globin gene, marked with a 6 nt deletion in the 5' untranslated region to allow its transcript to be distinguished from native  $\gamma$ -globin transcripts (Fig. 3). The globin gene was linked to a 400-nucleotide DNA fragment containing LCR site 2, and a bacterial neomycin resistance gene used for selection. Site 2 alone has been shown to confer high level globin gene expression in erythroleukemic K562 cells (see Regulation of Globin Gene Transcription) and when treated with hemin, these cells can be induced to differentiate and increase expression of  $\epsilon$ - and  $\gamma$ -globin genes (79).

We used the packaging strategy describe above (Production of the AAV Vector System) to generate AAV/globin hybrid virus. Recombinant virus was titered using human fibroblast target cells in the presence of geneticin (HeLa and/or Detroit 6) (78). Unconcentrated titers ranged from  $10^4$  to  $10^5$  neomycin-resistant colonies/ml. Southern analysis of low molecular weight DNA revealed no detectable wild-type particles using this protocol.

The erythroid cell line K562 was then infected with the recombinant virus and neomycin-resistant colonies were obtained (78). A polyclonal population of 30 isolated clones as well as individual clones were chosen for further study. First we characterized the configura-

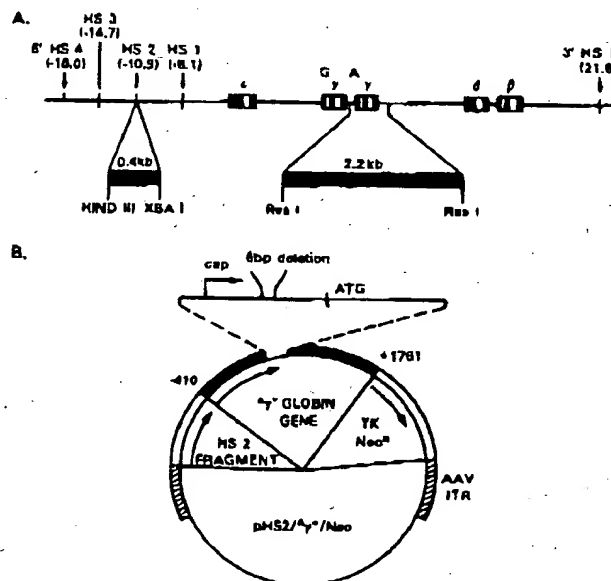


Figure 3. Construction of the rAAV/MS2/ $\gamma^A$ /Neo<sup>r</sup>-globin plasmid. (A) Schematic representation of the human  $\beta$ -globin cluster. The five functional genes are indicated. Arrows indicate the locations of the major DNase I sites. The MS2 fragment and the  $\gamma^A$ -globin gene used in the vector are shown. (B) MS2 fragment, marked  $\gamma^A$ -gene (with the 6 bp deletion) and the Neo<sup>r</sup> gene cassette subcloned into the recombinant AAV backbone.

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tion of the integrated AAV/globin genome. Digestion of genomic DNA with *PvuII* demonstrated an expected 1.2-kb globin fragment which corresponded to a single, unarranged proviral copy per cell. The K562 cell line, which is triploid at chromosome 11 (location of the globin cluster) by karyotype analysis (17), served as a control when estimating the AAV/globin copy number of individual clones. Individual clones digested and characterized as above revealed one to two copies of the transduced gene per cell. No rearrangement of the transduced gene was detected in either individual or pooled clones demonstrating the stability of the transduced gene.

As mentioned above, preliminary data from our laboratory indicates that AAV transducing vectors containing only the viral terminal repeats do not target to chromosome 19 (see Site-Specific Integration). We also probed genomic digests of the globin recombinants with chromosome 19-specific probe to further characterize the provirus integration in K562 cells. As expected, unlike wild-type AAV, which utilizes targeted integration, the AAV/globin viruses appeared to have integrated randomly.

RNAse protection assays demonstrated both basal (uninduced) and hemin-induced expression of the marked globin gene. Endogenous and transduced  $\gamma^A$  globin transcripts were identified as predicted bands of 145 nt and 117 nt, respectively. As shown in Figure 4, a strong signal at 145 nt represented transcription from the endogenous  $\gamma$ -globin genes present in K562 cell line. The probe measured both  $\gamma^A$  and  $\gamma^G$  endogenous transcripts. Assuming that all six endogenous copies of globin were expressed, we measured uninduced expression of the marked gene to be 70% that of a single endogenous gene which, with hemin induction, rose to 90%. Several non-erythroid tissue culture lines were examined for evidence of  $\gamma$ -globin transcripts. A small (1-5% of rAAV/K562 signal) but detectable signal was found in Detroit 6 and HeLa cells but not in T lymphoid CEM cells.

To further verify these results, messenger RNA expression was analyzed using the polymerase chain reaction (PCR). We determined previously the transcriptional start site of the marked  $\gamma$  globin gene in transduced K562 cells by a primer extension; PCR primers were designed accordingly. Cytoplasmic RNA isolated from either single or pooled K562 clones was reverse transcribed and amplified using primers that anneal to both endogenous and marked globin genes. The 6 nt deletion enabled resolution of these transcripts on 8% polyacrylamide-urea gels. Both uninduced and hemin-induced cells were analyzed, with mock-transduced K562 cells serving as a control. The marked transcript was detected in all clones tested.

Quantitation of RNA message by PCR was comparable to that determined by RNAse protection and

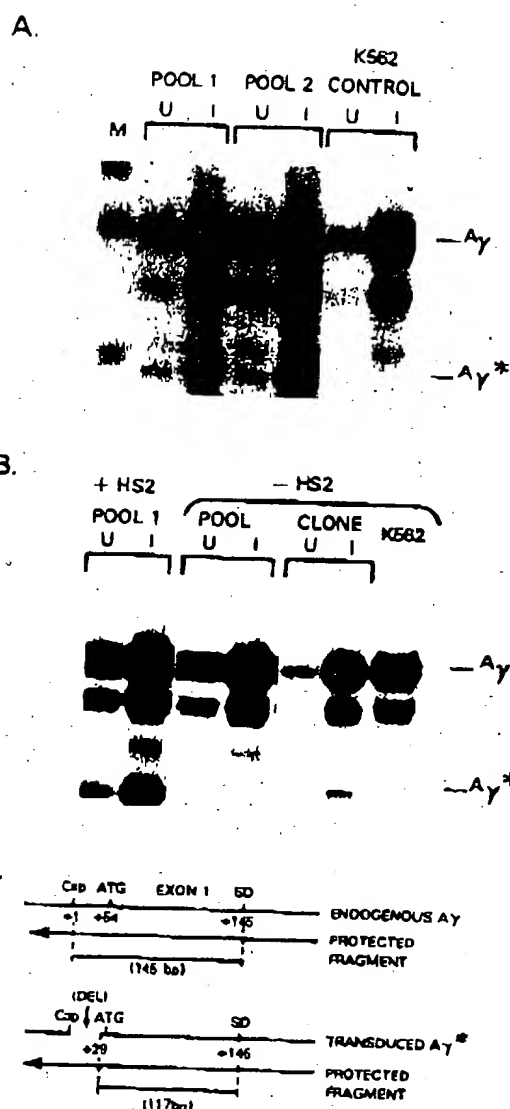


Figure 4. RNase protection assay of RNA extracted from K562 cells infected with (A) rAAV/HS2/ $\gamma^A$ /Neo<sup>r</sup> plasmid or (B) rAAV/ $\gamma^A$ /Neo<sup>r</sup>. (C) The expected RNA-protected fragments from the endogenous ( $\gamma^A$ ) and transduced ( $\gamma^*$ ) genes are shown. U, uninduced cells; I, hemin-induced cells. Refer to Ref. 78 for details.

confirmed high level expression of the transduced  $\gamma$  gene, including induction by hemin. S1 primer extension experiments confirmed that the correct globin start site was used in the proviral state. To rule out the possibility that the regulated globin expression we observed with this construct was due to globin regulatory sequences (LCR2) and not viral sequences or chromosomal positioning, we constructed an AAV/globin hybrid virus identical to the one described above minus the LCR2 site. Similar characterization of these transduced genes indicated a marked reduction in globin expression as expected.

The reduction of globin derived was observed in LCR site integrated clones. More reduced levels of transduction were observed.

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These results represent the first viral-based introduction in which correct levels and regulation of  $\gamma$ -globin gene expression were achieved in an erythroid-derived cell line. High level, regulated globin expression was obtained when using a construct containing the LCR site 2. The LCR/globin construct efficiently integrated into the genome without rearrangement in all clones studied. (Recent extensive data from our laboratory suggests approximately 10–20% rearrangement.) Moreover, the messenger RNA expression of the transduced gene was comparable to endogenous  $\gamma$ -globin levels. The correct globin start site was utilized in the transduced gene, and tissue-specific expression (which was hemin-inducible) was maintained.

The rAAV model can also be used to study the effects of specific mutations in the regulatory HS2 element on globin transcription (80). Specific mutations within the HS2 region of the rAAV/ $\gamma^A$ /globin vector described in Figure 3B were constructed, and neomycin-resistant clones containing the rAAV genome present in single copy were evaluated. Mutations within the NFE-2 binding motif resulted in a marked reduction of hemin-induced expression of the transduced globin gene when compared with expression from constructs containing the native HS2. In contrast, another set of mutations in the GATA-1 motif, which prevent binding of GATA-1, had no effect on basal and hemin-induced expression from the transduced globin gene. Other analyses of HS function rely on gene transfer methods which result in multiple copy integrants, and cooperative interaction between tandem gene copies are difficult to exclude. In this respect, the rAAV model is superior in that over half of the evaluable clones containing the native and mutant HS2 element had a single unrearranged copy of the rAAV genome. This single copy rAAV transduction model may also be useful for evaluating other regulatory elements and their effects on the transcription of genes linked *in cis*.

This suggests that recombinant AAV vectors can be used effectively for the transfer of globin genes into human cells. Previous work has demonstrated that AAV vectors are capable of transducing a selectable marker into murine hematopoietic progenitor cells (71). Moreover, we have generated and tested an AAV vector carrying B19 viral coding sequences for infection in erythroid progenitor cells from human bone marrow (81). B19 is an autonomously replicating parvovirus shown to be the etiologic agent of various clinical disorders in humans (hydrops fetalis, polyarthralgia syndrome, and transient aplastic crises associated with various hemolytic anemias). This parvovirus has so far been shown to replicate only in erythroid progenitor cells in human bone marrow. Using the recombinant AAV vector carrying the B19 coding sequences, rAAV stocks demonstrated that the AAV-based vector was capable of infecting human bone marrow cells (81).

Furthermore, the recombinant B19-AAV hybrids inhibited erythroid hematopoietic colony formation, indicating the expression of B19 genes.

We have recently demonstrated transduction of both rAAV and wild-type AAV human pluripotent cells (S. Goodman, unpublished results). rAAV carrying a  $\beta$ -galactosidase gene were capable of infecting CD34<sup>+</sup> selected bone marrow cells, as assessed by DNA PCR analysis of progenitor colonies derived from CD34<sup>+</sup> cells grown in methylcellulose. Incubation of wild-type AAV with CD34<sup>+</sup> selected cells demonstrated site-specific integration on chromosome 19q. rAAV vectors containing LCR-globin cassettes are currently being tested to transduce human and primate CD34<sup>+</sup> selected bone marrow cells. Gene transfer with rAAV into animal tissue has not yet been demonstrated.

#### Safety Issues

The safety of retroviral gene transduction has been reevaluated in murine and primate models. Most retroviral vector systems employ components of the Molony murine leukemia virus, known to induce T cell leukemia and lymphomas in mice (82). Initial investigations suggested no apparent pathologic effects of murine amphotropic replication-competent virus in primates (83, 84). However, a bone marrow transplantation/gene therapy experiment in primates using a high titer recombinant retrovirus contaminated with a moderate titer of wild-type retrovirus induced a rapidly progressive T cell lymphoma in three of 10 animals tested (85). Over 50–100 copies of the wild-type replication-competent provirus were detected in the tumor DNA, implicating viral insertional mutagenesis as the pathogenic mechanism. The use of newer retroviral packaging systems to reduce or eliminate wild-type retrovirus is an absolute necessity for human use (86).

One of the salient features of the AAV system is the lack of any demonstrable pathology to the host cell. As mentioned previously, no epidemiologic evidence currently exists linking AAV to human disease. The potential toxicity of rAAV in animals is unknown. Wild-type adenovirus, required for the generation of rAAV, is capable of causing disease in immunocompromised hosts. rAAV packaging systems will need to be modified to eliminate adenoviral contamination.

#### Summary

Gene transfer of human globin genes into human pluripotent stem cells via viral vectors may soon be realized. The high level of globin gene expression believed to be required for the treatment of severe hemoglobinopathies necessitated the inclusion of cis-acting sequences (LCR). Retroviral vectors containing the LCR elements are prone to rearrangement, low titer, and poor expression. Inclusion of a "minilocus" containing four HS sites linked to a globin gene resulted in

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higher expression in transplanted mice, but rearrangement of the provirus still occurs, and it is unclear what significance these experiments have with regard to human marrow stem cell transduction.

Recombinant AAV is among the newest of genetic transfer vectors. This once obscure virus possesses unique properties that distinguish it from all other vectors. Its major advantage is the lack of pathogenicity in humans. Wild-type AAV has the unusual ability to selectively integrate into the mammalian genome at a specific region, thus reducing the concern for genomic disruption and insertional mutagenesis. The ability of AAV to carry regulatory elements without interference from the viral template may enable greater control of transferred gene expression. Disadvantages currently include the inferior packaging systems which yield low numbers of recombinant virions which are contaminated with wild-type adenovirus. The small AAV genome that can be packaged (~5 kb) rules out its use for transfer of larger genes. Recombinant AAV viruses do not appear to demonstrate the same site-specific genomic integration as wild-type viruses. Elucidation of the mechanism of site-specific integration should prove useful in the development of safe vectors for gene transfer as well as provide insight into the nature of DNA recombination in humans.

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COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

IN THE MATTER OF: Australian  
Patent Application 696764  
(73941/94). In the name of:  
Human Genome Sciences Inc.

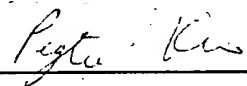
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IN THE MATTER OF: Opposition  
thereto by Ludwig Institute for Cancer  
Research, under Section 59 of the  
Patents Act.

Annexure GBC-21

This is **Annexure GBC-21** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
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**Gary Baxter Cox**

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Patent Attorney PEYTEE K H D O



# LYMPHATICS AND BLOOD VESSELS, LYMPHANGIOGENESIS AND HEMANGIOGENESIS: FROM CELL BIOLOGY TO CLINICAL MEDICINE

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## ABSTRACT

The past 15 years have witnessed an explosion of knowledge about blood vascular endothelium due in large part to in vitro growth of endothelial cells from both large blood vessels and capillaries. In contrast, little comparable information has accumulated on endothelium of lymphatics, which lie in intimate contact with parenchymal cells and drain excess fluid, macromolecules, particles, and immunocompetent cells in a continuous recirculation between tissues and bloodstream. While structural and functional differences between the two vascular systems have been described in vivo, in tissue sections, and in isolated preparations, similarities are notable in ultrastructure, biochemistry, physiology, and pharmacologic responsiveness, and these may predominate under pathologic conditions. In 1984, three separate groups described in vitro culture of lymphatic endothelial cells from collecting ducts and cavernous lymphangiomas. Lymphatic, like blood vascular, endothelium grows in confluent monolayers, "sprouts", synthesizes Factor VIII-associated antigen and fibronectin, and ultrastructurally shows Weibel-Palade bodies; overlapping intercellular junctions and anchoring filaments typical of lymphatic endothelium are also found. Genetic, congenital, and acquired disorders such as strangulating fetal nuchal cystic hygromas (Down and Turner syndromes), vascular tumors and dysmorphogenesis (Maffucci

and Klippel-Trenaunay syndromes), Kaposi's sarcoma, lymphogenous and hematogenous spread of cancer, and parasitic infestations such as filariasis, share overlapping abnormalities in formation, growth, and/or neoplasia of lymphatics and blood vessels. In these and similar clinical disorders, confusion often exists as to the nature of the cell or tissue of origin, and insight into the role and control of hemangiogenesis and lymphangiogenesis is still in its infancy. Nonetheless, with the ever widening array of investigative techniques, it is not only timely but imperative to explore the endothelial biology underlying these inborn and acquired disorders.

Blood and lymphatic vasculatures are closely intertwined in embryonic development and respond to many similar stimuli in the microenvironment (e.g. ischemia, inflammation, and neoplasia). The two circulations work together in an integrated fashion in the uptake and transport of interstitial liquid and macromolecules such as extravasated plasma proteins and ingested lipids, which recirculate between lymph, blood, and tissue. Distinct migration streams of immunocompetent cells interchange at various points in the "blood-lymph loop." Anatomic connections exist or open up between the two as lymphatic-venous communications, which function normally (viz. thoracic duct-jugular venous junction) or become operational under physiologic and pathologic conditions (e.g. carcinomatous

venous or lymphatic obstruction or in portal hypertension from alcoholic cirrhosis). While lymphatics closely resemble blood vessels on tissue section, they are more thin-walled attenuated structures containing bloodless fluid, and they ultrastructurally exhibit overlapping intercellular junctional complexes, specialized anchoring filaments, and discontinuous or absent basal lamina (1). Permeability, surface charge distribution, vesicular macromolecular movement, lipid absorption and transport, intrinsic contractility, and vasoresponsiveness of the two vasculatures are distinct in some respects, vary from organ to organ, and also may overlap.

The vascular endothelium is the crucial interface between circulating blood or lymph and the tissues. Two decades ago only surmised by Lord Florey to be more than an inert passive membrane or in "nucleated cellophane", endothelium is now recognized as the biologically active mentor of the microcirculation and of tissue homeostasis--originating, receiving, translating, transducing, and transmitting physical and chemical messages to and from different parts of the body. The ability to culture large and pure endothelial cell populations not only from major blood vessels but since 1979, also from human capillaries has produced an explosion of knowledge (1-3). Despite differences among species and organs, blood vascular endothelium (BVE) exhibits remarkably consistent morphology and function in vitro mimicking its structural, synthetic, and transport properties in vivo and in isolated vascular preparations. In culture, endothelial cells grow as confluent monolayers with characteristic cobblestone appearance, which under appropriate conditions sprout and form tubules, that is, display "angiogenesis in vitro." Distinctive ultrastructural features mirroring those found in tissue section include intricate intercellular junctions, micropinocytotic vesicles, intermediate filaments, and Weibel-Palade bodies, which are thought to manufacture or store Factor VIII-associated antigen (Factor VIII<sub>AA</sub>). On immunohistochemi-

cal examination, endothelial cells contain Factor VIII-associated antigen, angiotensin-converting enzyme, and extracellular matrix components such as fibronectin, all of which in addition to prostacyclin and many other vasoactive metabolites can be measured quantitatively after release into the supernatant overlying the monolayer.

Considerable attention has been directed to a search for angiogenic factors controlling blood vessel formation and thereby tumor and organ growth. The genetic code for one such substance, angiogenin, has recently been deciphered. Blood vascular endothelium interacts in a "symbiotic" relationship with immunocompetent cells, directing lymphocyte cell traffic and "homing" and also producing colony-stimulating activity differentiating hemopoietic stem cells into granulocytes and monocytes (4). Immunocompetent cells as well as fibroblasts and adipocytes in turn secrete angiogenic factors and share cell surface receptors with endothelium. Thus, in large part because blood vascular endothelium can now be isolated *in vitro*, its role as a structural barrier as well as active facilitator of small solute and macromolecular transport into and out of tissues, as a director of cellular migration, as a stabilizer of the coagulation cascade, and as a biosynthetic factory is now being unraveled. Blood vascular endothelial damage and/or repair have been implicated in processes as diverse as atherosclerosis, hypertension, inflammation, wound healing, ischemia, diabetes mellitus, and transplant rejection.

Yet, in part because of a lack of analogous *in vitro* models, only rudimentary information is available about the highly permeable vascular interface on the "dark side" of the blood capillary barrier, deep in the tissues, separating circulating "lymph" from extracellular matrix and liquid and parenchymal cells in lymphoid and non-lymphoid tissues. Within this oft forgotten sluggish lymphatic-tissue fluid circulation pass surplus liquid, macromolecules, particles, and migrating cells from the interstitium on their way

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Fig. 1. Schematic diagram of a blood vessel cross-section showing the relationship between the vessel wall and the lumen.

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through regional and central lymph nodes before returning to the blood circulation (Fig. 1). In 1984 for the first time,

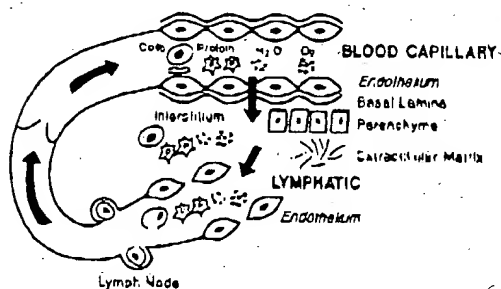
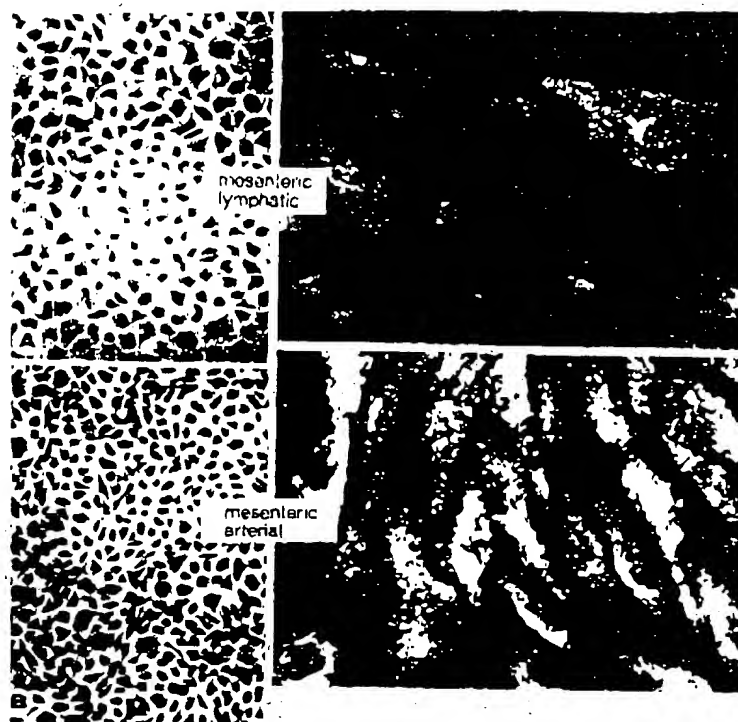


Fig. 1. Blood-lymph loop. There is a continuous circulation and re-circulation of cells, particles, macromolecules, small solutes, and gases between blood, tissues, and lymph. Blood vascular and lymphatic endothelium represent crucial interfaces in this circulation. Lymph exiting parenchymal organs filters through lymph nodes on its way to return thru lymphatic-venous communications to the bloodstream.

small relatively pure populations of lymphatic vascular endothelium were isolated by our group from explants of a massive recurrent lymphangioma (5), by Johnston from normal bovine lymphatic ducts (6) and by Gnepp from canine and human cadaveric thoracic ducts (Figs. 2 and 3) (7). In culture, lymphatic vascular endothelium (LVE) from large ducts and microvasculature bears a strong resemblance to BVE, forming confluent "cobblestone" monolayers, sprouting (Fig. 4), and staining positively for endothelial markers, Factor VIII<sup>AA</sup> and Ulex lectin. Thus, C113, our second nearly pure lymphatic endothelial cell line from a recurrent chylous retroperitoneal lymphangioma resembles blood vascular endothelium in staining positively for Factor VIII<sup>AA</sup>, F-actin, and fibronectin (Fig. 5), and exhibiting numerous Weibel-Palade bodies (Fig. 6L) (8). Nonetheless, LVE appears to possess some distinctive features: overlapping intercellular junctions and abundant intermediate anchoring-type filaments typical of lymphatic endothelium (Fig. 6R) (8). Although questions may be raised about stromal blood vessels giving rise to some of the endothelial cells found in this

lymphangioma cell line, the same question can be but has not been raised about designated blood vascular endothelial cell cultures from microvasculature in such standard sources as omentum and foreskin, tissues rich in lymphatics as well as blood vessels. At this point, there is every reason to believe that lymphatic like blood vascular endothelium is also a vast endocrine organ maintaining a lymph-fluid compatible surface and a changeable selective interface between the lumen and interstitium that is also the target for numerous perturbations affecting not only its intrinsic structure and function but also that of surrounding tissues.

The close interactions between the lymphatic and blood vasculature and lymphangiogenesis and hemangiogenesis on a cellular and organ level are further illustrated in the clinical manifestations of disease. Congenital lymphologic syndromes of genetic or intrauterine origin involving abnormal growth of lymphatics often include widespread blood vascular abnormalities as in Maffucci's and Klippel-Trenaunay syndromes (Figs. 7 and 8). These soft tissue hemangiomas and lymphangiomas are commonly accompanied by lymphedema, venous aplasia or hypoplasia as well as arteriovenous anomalies and striking soft tissue overgrowth such as limb hypertrophy and macrodactyly, likely closely linked to the circulatory disturbances (Figs. 7 and 8). On rare occasions malignant vascular transformation may take place. Blood vascular and lymphatic anomalies also coexist in Turner's XY gonadal dysgenesis syndrome where webbed neck from regressed fetal cystic lymphangiomas, extremity lymphedema associated with lymphatic hypoplasia and aplasia, and coarctation of the aorta are typical manifestations; variants of the syndrome also exhibit severe intracardiac anomalies. Down syndrome (trisomy-21) similarly may present in utero with strangulating cystic hygromas, cardiovascular anomalies and lymphedema or survive into adulthood with other variations of these vascular abnormalities. On the other hand, in acquired condi-



## BOVINE MESENTERIC VASCULAR ENDOTHELIUM

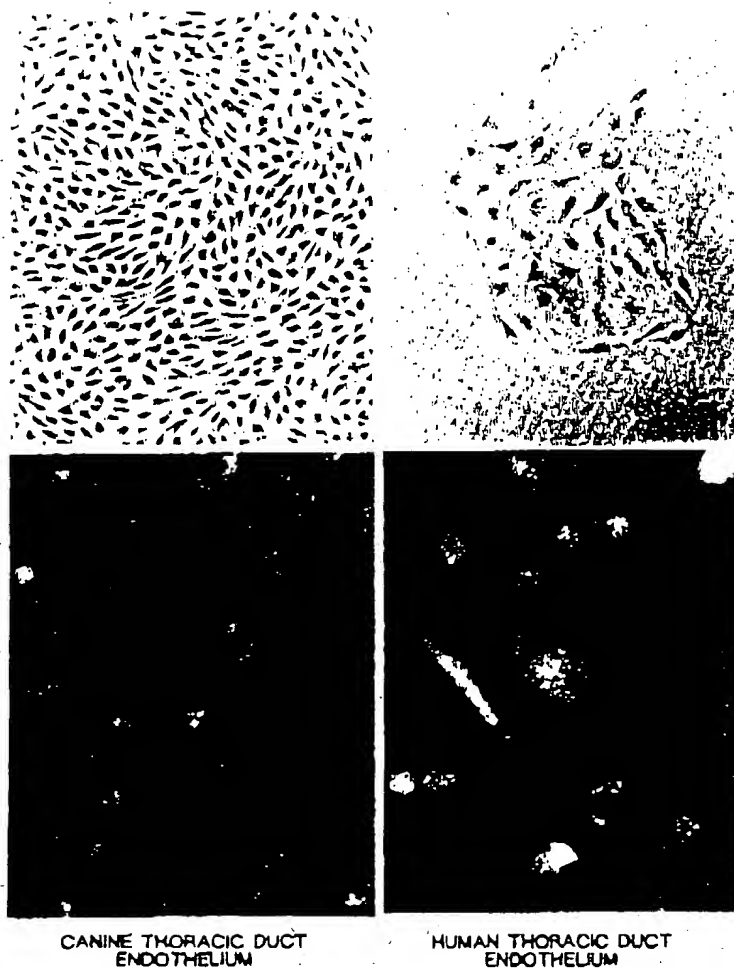
(M.G. Johnston and M.A. Walker, *In Vitro*, 1984)

Fig. 2. Comparison of bovine lymphatic endothelial with bovine superior mesenteric arterial endothelial in tissue culture. The cells in A and B were stained with a Hemacolor stain kit (Harleco) as follows. The cells were washed twice with PBS and fixed for 5 min in methanol. The cells received an eosin solution (30s) followed by a thiazine solution (30s) and were then washed with water. A, Bovine lymphatic endothelial cells, Passage 9; (x141). B, bovine mesenteric artery endothelial cells, Passage 4; (x141); (6; modified by permission). C and D show assay for Factor VIII-related antigen. C, bovine lymphatic endothelium. D, Bovine mesenteric artery endothelium (antibody to human Factor VIII-related antigen diluted 1/10) (6; modified by permission).

tions such as classical or AIDS-associated epidemic Kaposi's sarcoma, abnormal lymphatic-venous communications may comprise or contribute to the multicentric tumor some of its peculiar morphologic and immunohistochemical properties as well as the associated lymphedema and hemorrhage. On rare occasions, malignant vascular tumors appear as Stewart-Treves syndrome after many years of lymphostasis associated with intense he-

mangiogenesis and lymphangiogenesis, and lymphangiomatoid changes superimposed on exuberant profuse scarring and fat deposition. The latter is well exemplified in filarial infestation, which leads to elephantiasis where thickening and piling up of the lymphatic as well as blood vascular endothelium, intraluminal blood or lymph clots, and exuberant deposition of underlying scar tissue characterize the pathologic process and the

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(D. Gnepp and W. Chandler, *In Vitro*, 1985)

*Fig. 3. L, upper, canine thoracic duct culture demonstrating a sheet of uniform contact inhibited non-overlapping endothelial cells with typical cobblestone morphology. (x131). L, lower, indirect immunofluorescence of canine thoracic duct endothelial cells demonstrating marked positivity of Factor VIII antigen (note granular cytoplasmic staining. (x189). R, upper, human thoracic duct culture demonstrating one nest of typical endothelial cells, Day 9. (x129). R, lower, indirect immunofluorescence of human thoracic duct endothelial cells demonstrating granular cytoplasmic and perinuclear fluorescence. (x189). (7; used by permission).*

host response to the worm and its products. These interrelationships between lymphangiogenesis and lymphologic syndromes have been summarized by us recently (9), and an analogous intercon-

nected scheme can be postulated for hemangiogenesis and blood vascular syndromes.

Endothelial biologists working in tissue culture have opened up the pheno-

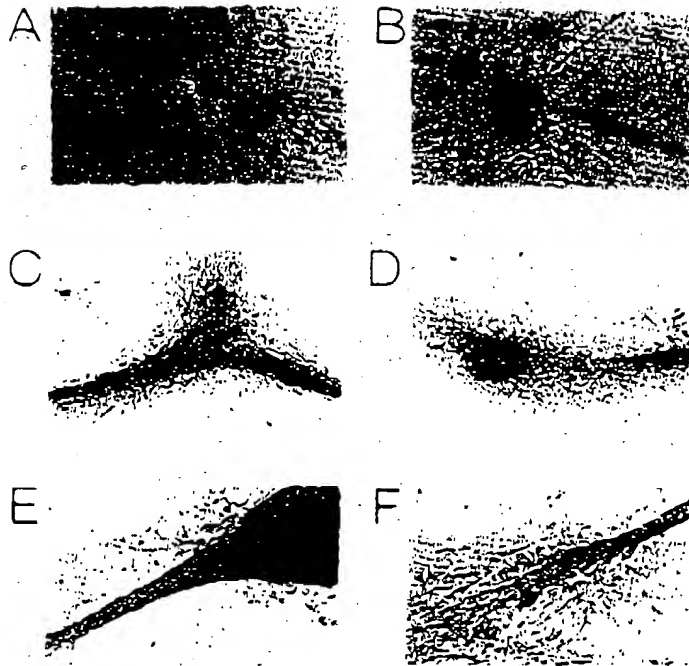


Fig. 4. Photomicrographs (inverted light) depicting in vitro evolution of lymphatic endothelial cell growth derived from a resected lymphangioma of the knee. An "early" phase displays a loose cluster of endothelium with occasional "sprouting" or branching (A:  $\times 100$ ). With time, this sprouting pattern becomes more prominent (B;  $\times 100$ ) and eventually evolves into a sheet-like cellular aggregate (C,D:  $\times 40$ ) with intense "lymphatic-like" tumorous branching (E,F:  $\times 100$ ).

menon of tumor angiogenesis to intensive inquiry. In 1972, Folkman (10) first proposed the concept that all tumors are angiogenesis-dependent and once tumor take has occurred, enlargement of the tumor cell population is preceded by growth of new blood capillaries converging on the tumor. Inhibition of angiogenesis, he proposed, might be a therapeutic approach to solid tumors. Interesting questions have arisen about the role of endothelial mitogens in normal tissues and natural mechanisms that restrain and inhibit formation of capillary and thereby tissue and organ growth. Lymphatic vessels have been scarcely mentioned in the context of "angiogenesis", and some workers have even suggested that tumors do not contain lymphatics. Nonetheless,

the parallel development and common response of the two vascular systems to varied physiologic and pathologic stimuli suggest, however, that hemangiogenesis and lymphangiogenesis go hand in hand and that the mysterious growth factors stimulating both vasculatures are self-generated as well as arise in or are delivered through the neighboring tissue matrix; that is, the stimuli are autocrine, paracrine, and endocrine.

Despite the mounting interest in tumor angiogenesis, investigation of a related process--what we have termed "angiotumorigenesis," i.e., the growth and development of blood and lymphatic vascular tumors--has been extremely limited despite their frequency as cosmetic imperfections or as disfiguring or even



Fig. 5. Internal pattern of Factor XIIIa-stained cytoplasmic fibrinogen in a tumor.

life-threatening. Genetic, environmental influences, and by hormonal control (e.g., vitamin D, e.g., human cytomegaly virus) but these endothelial tumors, continues at a tumors, rests (hormone more expression. As areas of strikingly normal wildly available as Moreover tumors multiten sionally, tumors and even sizing in hand, but tumors Unfortun



Fig. 5. Indirect fluorescent-antibody labeling using rabbit anti-human IgG shows the characteristic granular pattern of Factor VIII-related antigen (A) ( $\times 168$ ), F-actin positive microfilament bundles forming a well-organized cytoskeleton stained with NBD-phalloidin ( $\times 591$ ), cell surface-associated fibronectin using rabbit anti-human fibronectin IgG (C) ( $\times 168$ ), which is also deposited extracellularly (8; used by permission).

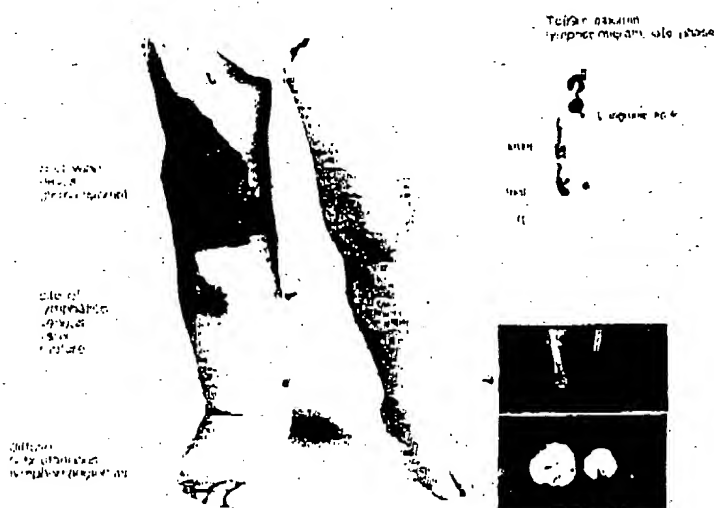
life-threatening tumors of childhood. Genetic, congenital and environmental influences on endothelial growth, such as by hormones and drugs (e.g., estrogens, oral contraceptives), industrial carcinogens (e.g., vinyl chloride) and viral agents (e.g., human immunodeficiency virus and cytomegalovirus) are poorly understood but these agents are known stimulants of endothelial proliferation. DNA transformation, and neoplasia. Controversy continues about the nature of some vascular tumors, i.e., whether they are embryonic rests (hamartomas), true neoplasms or mere expressions of exuberant angiogenesis. As in Kaposi's sarcoma, different areas of the same lesion may appear strikingly heterogeneous ranging from normal vessels to highly anaplastic or wildly aberrant structures indistinguishable as lymphatics or blood vessels. Moreover, it is unclear whether multiple tumors in separated or remote sites are multicentric in origin or metastatic. Occasionally, benign-appearing endothelial tumors may exhibit local invasion, recur, and even spread (e.g., "benign metastasizing lymphangioma"). On the other hand, benign and even malignant vascular tumors sometimes spontaneously regress. Unfortunately, immunohistochemical

studies to detect the presence of intracytoplasmic or cell surface endothelial markers (e.g., Factor VIII<sup>AA</sup> or basement membrane components) have produced more confusion than clarification because of heterogeneity of staining, postulated but disputed differences between the blood and lymphatic vasculature, inconsistent staining techniques, neoplastic or non-neoplastic transformation to more primitive or aberrant cell types or pluripotent nature of the cells, and presence of mixed cell types including mesenchymal and lymphoid elements. Thus, in part because of the paucity of animal models and the lack of *in vitro* systems to study pure populations of tumor cells, classification of endothelial tumors of lymphatic or blood vascular origin remains largely based on morphologic criteria and clinical behavior. Although tumor modulation by hormones, immunoregulatory substances, and growth factors seems almost within our grasp, detection and treatment of these neoplasms has progressed little over the past several decades beyond refinement or extension of surgical resection.

Understanding the structural-functional interrelationships between the blood and lymphatic vasculatures *in vivo*



Fig. 6. Transmission electron microscopy (A-D) of cultured tumor cells reveals relatively smooth cell surfaces with few microvillous projections, numerous vesicles, and cytoplasm rich with Golgi and rough endoplasmic reticulum. Abundant Weibel-Palade bodies (A, arrow; B, higher magnification) are seen surrounded by bundles of intermediate filaments characteristic of lymphatic endothelium. Higher power detail of intermediate filaments can be best appreciated in C. Typical macula adherens, overlapping intercellular junctions are also shown (C,D). (A= $\times 8550$ ; B= $\times 41,800$ ; C= $\times 48,450$ ; D= $\times 26,650$ ) (8; used by permission).



#### KLIPPEL TRENAUNAY SYNDROME

Fig. 7. 3-year-old girl with Klippel-Trenaunay syndrome involving the right leg. In addition to the characteristic port wine nevus and agenesis of the deep venous system, small pedicled arteriovenous fistulae lymphoscintigraphy ( $^{99m}\text{Tc}$  albumin) shows aberrant lateral "pick-up" with a large, dilated truncal "lake" reaching the lateral groin only after many hours. The left leg is unremarkable. Computed tomography with contrast enhancement suggests diffuse lymphangiomatous change with little or no edema fluid in the right leg.

Fig. 8. 13-year-old girl with Klippel-Trenaunay syndrome. Present are right leg.

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Fig. 8. 13-year-old girl with multiple lymphangomas including of the back, mediastinum and left axilla. Also present are right arm hypertrophy, macrodactyly, digital hyperemia.

and *in vitro* is key to unraveling the control mechanisms and detailed steps of tissue growth and repair from embryonic life to senescence. Greater availability and variety of *in vitro* models of normal and neoplastic endothelium should provide clues to detection of abnormal lymphangiogenesis and hemangiogenesis (e.g., enhanced or inhibited release of distinctive endothelial products from neoplastic endothelium) as well as more effective treatment (such as by angiostatic and angiostatic agents including naturally occurring hormones for chemo- and radioresistant vascular tumors or excessive scar formation. Isolated in pure culture, released from central control and tele-scoped in time, such *in vitro* models combined with the tools of molecular biology can also be used to explore endothelial cell interactions with related and distant cell types thereby potentially shedding light on disorders as varied as transplant rejection, inflammation, hypoplastic lymphedema, scleroderma, diabetes mellitus, lymphogenous vs. hematogenous spread of cancer, and limb ischemia.

What these test tube models teach us about fundamental biology must then be returned to the body, validated, and applied to this bewildering array of human disorders characterized by defective, exuberant, or uncontrolled hemangiogenesis and/or lymphangiogenesis and a wide variety of interrelated and dependent phenomena.

#### ACKNOWLEDGEMENTS

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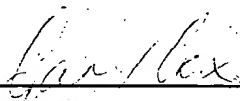
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Thirteenth day of December 2000.

  
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**Gary Baxter Cox**

WITNESS:

  
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## Supplement

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## A comparative study of cultured vascular and lymphatic endothelium

By L. C. J. YONG and B. E. JONES

With 11 figures

Received: July 27, 1989; Accepted: October 3, 1989

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**Keywords:** endothelium, lymphatic; lymphatic endothelium, cultured; factor VIII related antigen; antigen, factor VIII related; Weibel-Palade bodies; aortic endothelial cells

### Summary

There is comparatively little knowledge of the structure and function of *cultured* lymphatic endothelium. A study was carried out to compare the intrinsic growth characteristics of cultured lymphatic endothelial cells with cultured endothelia derived from blood vessels. It was found that cultured *lymphatic endothelium* has growth requirements and growth characteristics similar to vascular endothelium. It also possesses FVIII-RA and Weibel-Palade bodies for specific identification. The results of this study have provided important base line data for subsequent studies of the pathobiology of lymphatic endothelium.

### Introduction

*In vitro* studies in the last 10 years have mainly focused on studies of endothelium derived from blood vessels (6). Such studies greatly advanced our understanding of the role that blood vascular endothelia play in blood homeostasis, inflammation, tumour angiogenesis and atherogenesis. *In vitro* studies have established that vascular endothelial cells are site and organ specific, produce a whole range of active substances, and have specific markers for their identification (9). There are no studies of cultured lymphatic endothelium on a comparable scale even though it is generally accepted that lymphatic vessels play important roles in various pathobiological processes such as inflammation, tumour spread and immunologic reactions. Lymphatic vessels are difficult to visualise in ordinary tissue sections and functionally regarded as passive conduits for returning excess fluid from the interstitial tissue to the main circulation, but how they play an active role in the spread of malignant tumours, inflammation, healing and regeneration as well as immunological reactions is not clear. There is little doubt that *in vitro* studies of lymphatic endothelium on a similar scale to vascular endothelium will help to define more specific roles that lymphatic vessels may play in various pathobiological processes.

The techniques for the culture of endothelia from blood vessels are well established (6). Evidence from a few preliminary reports suggests that successful cultures of lymphatic

endothelium could be achieved from the same techniques for the culture of vascular endothelium (9, 10). As a first step towards a subsequent study of the immunological and secretory characteristics of cultured lymphatic endothelium, we set out to establish cultures of lymphatic endothelium, study their growth and structural characteristics and compare them with cultures of endothelium from blood vessels. The results from this study will provide valuable base line data for subsequent studies of the pathobiology of lymphatic endothelium.

## Materials and Methods

### *Isolation of endothelial cells from bovine mesenteric lymphatic vessels*

Fresh bovine mesentery was obtained from the local abattoir. The mesenteric vessels were identified by injecting a solution of 0.1% Evan's blue dye in phosphate buffered saline (PBS) into a mesenteric lymph node. The dye filled segment was ligated and dissected free from the adipose tissue. The lymphatic vessel was cannulated at both ends and gently flushed with Hank's balanced salt solution (HBSS). The lymphatic was then filled with HBSS containing 1.5 mg/ml of collagenase (Boehringer Mannheim) and 0.2% foetal calf serum (FCS) and incubated at 37 °C for 10 min. The lymphatic vessel was then drained and the fluid collected. The vessel was flushed with an additional 20 ml of HBSS. The total volume of solution was centrifuged at 3,000 rpm for 15 min. The cell pellet was resuspended in 1 ml of complete culture media and placed in one well of a Linbro multiwell plate (9.62 cm<sup>2</sup>). A further 2 ml of culture media was added to the culture well. Usually the cells from 2 lymphatic vessels were pooled and placed in one culture well.

### *Isolation of bovine aortic endothelial cells*

Fresh aorta obtained from the local abattoir was dissected free of all fibrofatty tissue and the branches ligated. The aorta was then filled with HBSS containing 1.5 mg/ml of collagenase and 0.2% FCS and incubated at 37 °C for 10 min. The perfusate was recovered, centrifuged and the pellet resuspended in 1 ml of HBSS and placed in culture well with an additional 2 ml of complete culture media.

### *Isolation of human umbilical vein endothelial cells*

Human umbilical cords were obtained from the associated obstetrics hospital of the University of New South Wales. The endothelial cells were isolated from the vein using established techniques of collagenase dispersion. Briefly, the umbilical vein was inspected for damage and cannulated at both ends and rinsed with HBSS and then filled with 0.1% collagenase containing 0.2% FCS and incubated for 10 min at 37 °C. The solution was then collected and the vein flushed to remove residual endothelial cells. The cells were concentrated into a pellet by centrifugation at 1,000 rpm for 5 min, resuspended in 1 ml of complete culture media and placed in culture well (7).

### *Culture conditions*

All primary and subcultures were grown in modified Medium 199 containing Earl's salts, 20 mMol HEPES buffer and glutamine supplemented with 20% FCS (Flow), 100 µg/ml endothelial cell growth supplement (ECGS), 100 µg/ml heparin (Sigma), penicillin 50 µg/ml (Flow), streptomycin 50 µg/ml and fungizone 2.5 µg/ml (Flow).

All culture flasks and wells were coated with 1% gelatin (Sigma) in Ca<sup>++</sup> and Mg<sup>++</sup> free PBS. Primary cultures were grown in Linbro multiwell plates and subcultures were grown in Nunclon 25 cm<sup>2</sup> flasks at 37 °C in a humidified incubator with air and 5% CO<sub>2</sub>. Cells were passaged and subcultured by standard techniques of trypsin digestion using 0.25% trypsin, 0.02% EDTA in PBS. Cells were passaged at a ratio of 1:3 and subcultures reached confluence in 7 days. Half the culture medium was replaced in all cultures at second daily intervals.

### *Isolation and culture*

Human aortic from head injury, described in the p  
Capillary endo from circumcision squeezed with a su were removed by during culture. Ci Medium 199, 100

### *Identification of e*

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### *Proliferation Sina*

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### *Preparation of cu*

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Cultured cells Glass coverslips mately 2-2.5 x 1 Cells were then f

### *Normal lymphatic*

Normal mese mission and scan cells.

#### *Isolation and culture of human aortic and capillary endothelial cells*

Human aortic endothelium was cultured from thoracic aorta of young adults after death from head injury. Endothelial cells were harvested by the techniques of enzyme dispersion as described in the preceding sections.

Capillary endothelial cells were isolated and cultured from the neonatal foreskin obtained from circumcision. Briefly foreskin was cut into small pieces and after 0.3% trypsin digestion squeezed with a scalpel blade to extrude endothelial cells from capillaries. Contaminant cells were removed by centrifugation in Percoll and by mechanical weeding with sterile glass probe during culture. Cultures of capillary endothelium were maintained in 20% human serum in Medium 199, 100 µg/ml ECGS and 100 µg/ml heparin.

#### *Identification of endothelial cells*

It is generally accepted that the best method of identification of endothelial cells is the detection of factor VIII related antigen (FVIII:RA) which can be readily demonstrated by immunohistochemical techniques (8). All cultured cells in this study were subjected to indirect immunofluorescent microscopy for expression of FVIII:RA. Cells were grown on coverslips for 4–5 days and removed from culture flasks, rinsed in PBS, fixed in cold acetone and air dried. The cover slips were then washed in Triton X100 (0.3% in PBS), rinsed in PBS and incubated with primary antibody, rabbit anti-human FVIII:RA at a dilution of 1:100 in PBS with 3% pig sera for 1 h. The coverslip was then rinsed in PBS and incubated with FITC labelled swine anti-rabbit IgG with 3% pig sera for 30 min. The cover slip was then washed and mounted with glycerol/PBS (1:9), examined and photographed.

#### *Proliferation Studies*

The rate of cellular proliferation was determined by estimation of the *doubling time* which is the time taken for cell cultures to double their population in a given culture condition. Cells were grown in Linbro multiwell plates of 2.0 cm each. Each well was inoculated with approximately  $5.8 \times 10^4$  cells/cm<sup>2</sup>. The number of cells in each well was counted in a haemocytometer after 24, 48 and 72 h intervals. The cell counts were plotted on a semilog graph and doubling times determined by analysis of the growth curves (7).

#### *Preparation of cultured cells for electron microscopy*

Cultured cells were prepared for transmission electron microscopy by established techniques of HAUDENSCHILD *et al.* (5). Cells grown in Nunclon flasks were fixed *in situ* with freshly prepared 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 1 h and processed by standard techniques. Toluidine blue (0.1%) was added to identify cell colonies during each step of processing. After processing blocks of flask with attached cell layer were selected and sectioned for examination with a Philips 300 electron microscope.

Cultured cells were prepared for scanning electron microscopy using standard techniques. Glass coverslips were inoculated with 50 µl aliquot of cell suspension containing approximately  $2-2.5 \times 10^4$  cells and placed in a petri dish containing culture media for 4–5 days. Cells were then fixed in glutaraldehyde.

#### *Normal lymphatic vessels*

Normal mesenteric bovine lymphatic vessels were prepared for light microscopy, transmission and scanning electron microscopy by standard methods for comparison with cultured cells.

## Results

### General growth characteristics of cultured endothelial cells

The techniques used for the isolation and culture of vascular endothelial cells and the growth characteristics of bovine aortic endothelium and human umbilical vein endothelium are well documented (2, 6, 14). The characteristic features of vascular endothelium compared with those of lymphatic endothelium are presented in table 1. It was found that lymphatic

**Table 1.** Growth and structural characteristics of cultured endothelial cells.

Cell type	Cytology	Growth pattern	Ultrastructural features		Immunohistochemistry		Proliferation (doubling time)
			WBP	Tight jns	FVIIIIRA	UEA-1	
HUVE	Polygonal	Monolayer	+++	++++	+++	+	14h
HAE	Polygonal	Monolayer	+++	+++	+++	+	30-40h
BAE	Polygonal	Monolayer	-	++	+++	+	30-40h
BLE	Polygonal	Monolayer	++	+	+++	++	16h

WBP: - = absent; + = present; ++ = frequent; +++ = numerous; FVIIIIRA: - = absent; +++ = 100% positive; UEA-1: - = absent; + = faint; ++ = bright staining

endothelial cells were readily detached and dispersed from the vessel wall using a collagenase solution containing 1.5 to 2.0 mg/ml of enzyme. A higher concentration of collagenase appeared to cause detachment and disruption of contaminant cells as well as endothelial cells. A layer of gelatin was also essential for growth of lymphatic endothelial cells. Cells were attached to the gelatin-coated surface of the culture vessels within 3 h of inoculation. By 2-3 d (fig. 1) proliferative activity was well in advance and reached confluence by 5 to 7 days. Associated with endothelial cells were elongated fibroblastic cells. They were eliminated by a process of mechanical weeding using sterile glass probe under phase contrast microscopy. Under these conditions relatively pure cultures of lymphatic endothelial cells were obtained. Cultured vascular and lymphatic endothelial cells at confluence attained cell densities ranged from  $2.7 \times 10^4$  cells/cm<sup>2</sup> to  $7.4 \times 10^4$  cells/cm<sup>2</sup>. The average cell density for each cell type was highest for Bovine aortic endothelium (BAE) at  $4.2 \times 10^4$  cells/cm<sup>2</sup>, Bovine lymphatic endothelium (BLE) at  $3.8 \times 10^4$  cells/cm<sup>2</sup> and human umbilical vein endothelium (HUVE) at  $3.5 \times 10^4$  cells/cm<sup>2</sup>. The cell density did not appear to significantly decline with prolonged culture although some large multinucleated cell forms appeared in older cultures. The cells did not increase in size with repeated sub-cultures.

Cultured cells were examined by indirect immunofluorescent microscopy and for FVIIIIRA. Cryostat sections of normal lymphatic vessels were also examined for FVIIIIRA expression by indirect immunofluorescence.

### Ultrastructural features of cultured lymphatic endothelial cells

To our knowledge, there are no detailed reports of the ultrastructure of cultured lymphatic endothelium. The method of preparation of monolayers of cultured cells for ultrastructural studies ensured that the integrity and intercellular as well as the cell/substrate relationship of

**Fig. 2.** Cultured bovine lymphatic endothelial cell. Note Weibel-Palade body (WBP) in close proximity to cisterna (arrow) of Golgi apparatus (G).  $\times 71,000$ .



**Fig. 1.** Cultured human lymphatic endothelial cells showing elongated morphology.



endothelial cells and the  
 ilical vein endothelium are  
 ar endothelium compared  
 was found that lymphatic  
 ial cells.

histo- ry	Proliferation (doubling time)
UEA-1	
+	14 h
+	30-40 h
+	30-40 h
++	16 h

ous; FVIIIIR: - = absent;  
 bright staining

sel wall using a collagenase  
 ncentration of collagenase  
 as well as endothelial cells.  
 endothelial cells. Cells were  
 h of inoculation. By 2-3 d  
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 helial cells were obtained.  
 tained cell densities ranged  
 l density for each cell type  
 ills/cm<sup>2</sup>. Bovine lymphatic  
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 cell/substrate relationship of

Palade body (WPB) in close

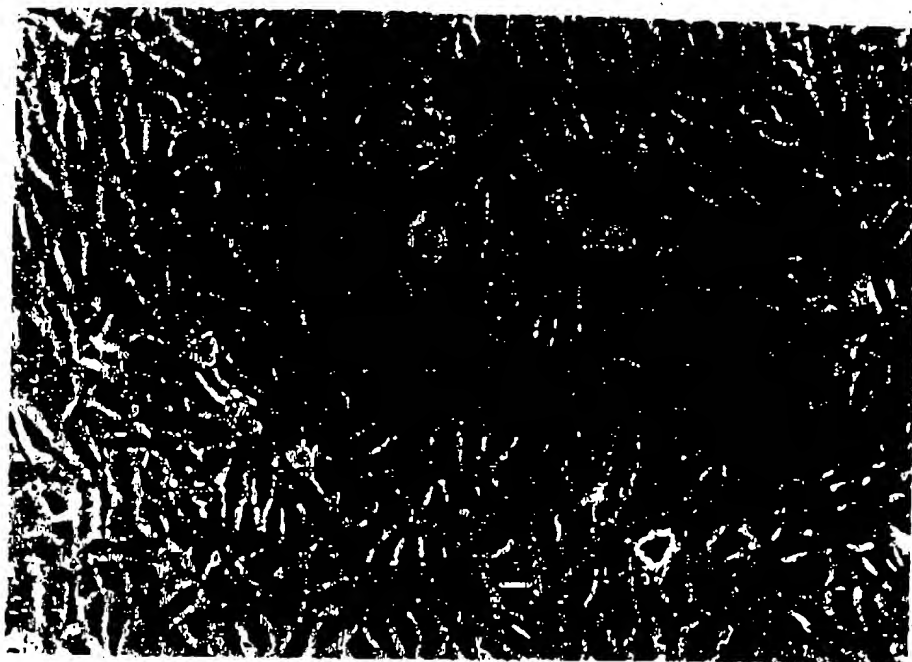
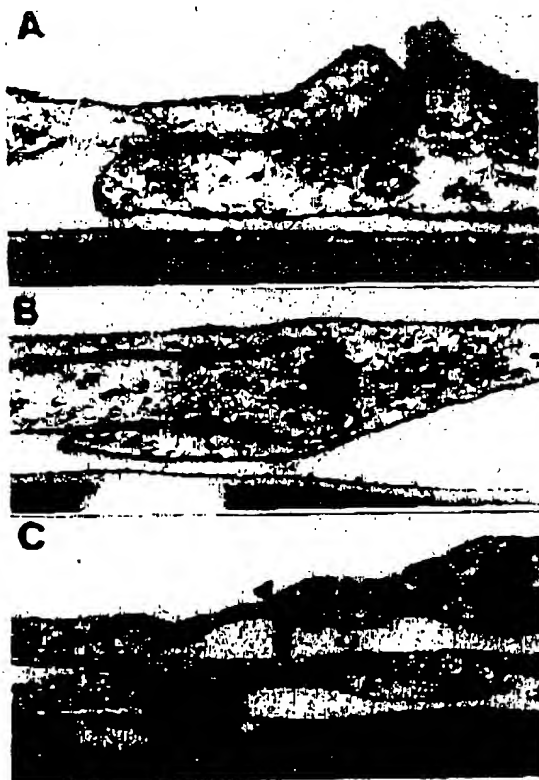


Fig. 1. Cultured human umbilical vein endothelial cells after 3 days showing polygonal and elongated morphology. Phase contrast  $\times 350$ .



in card statement





**Fig. 3.** Different types of intercellular junctions of cultured endothelial cells. A = simple overlapping of adjacent cells.  $\times 94,000$ . B = Mortise-like junction.  $\times 74,000$ . C = tight junction, rarely seen in cultured lymphatic endothelium.  $\times 28,000$ .

the monolayer was maintained. Cultured endothelial cells covered the gelatin surface as a distinct monolayer at confluence. The cells have central nuclei with cell borders extending over considerable distances in all directions. Cells were bound by a distinct cytoplasmic membrane with invaginations and often narrow openings forming caveolae. There were also numerous randomly placed surface projections most of which have club-shaped ends similar to those seen in vascular endothelium. The cytoplasm contained the usual cytoplasmic organelles including mitochondria, ribosomes, rough endoplasmic reticulum, Golgi apparatus, filaments and vesicular bodies. The Weibel-Palade (WPB) or specific endothelial granule was frequently seen in the cytoplasm, sometimes situated in a position close to the Golgi apparatus (fig. 2). The WPB had a specific internal substructure characterised by a series of longitudinal fine tubular structures arranged in a parallel fashion to the longitudinal axis. The appearance of the WPB varied considerably depending on whether it was cut in cross or oblique sections or whether the section transected the broad or narrow end of the club-shaped structure. Consequently the WPB could be easily mistaken for mitochondria. Cultured lymphatic endothelial cells maintained a close intercellular relationship. There were 3 types of intercellular junctions in vascular and lymphatic endothelial cultures (fig. 3). The most common type was a simple overlapping of one cell on another. The less commonly encountered type was the



**Fig. 4.** Surface rough mitochondria (MC) and (arrow heads) interdigitating junctions which were locked membranes.

interdigitating junctions which were locked membranes. The ultrastructural of cultured lymphatic endothelial cells as well as WPB were also observed.

#### *The ultrastructure of cultured lymphatic endothelial cells and human capillaries*

There was general agreement that the ultrastructure of cultured lymphatic endothelial cells from different sources was summarised in tabular form. The cytoplasm contained rough and smooth endoplasmic reticulum, Golgi apparatus, and the cell surface showed exocytic vesicles.

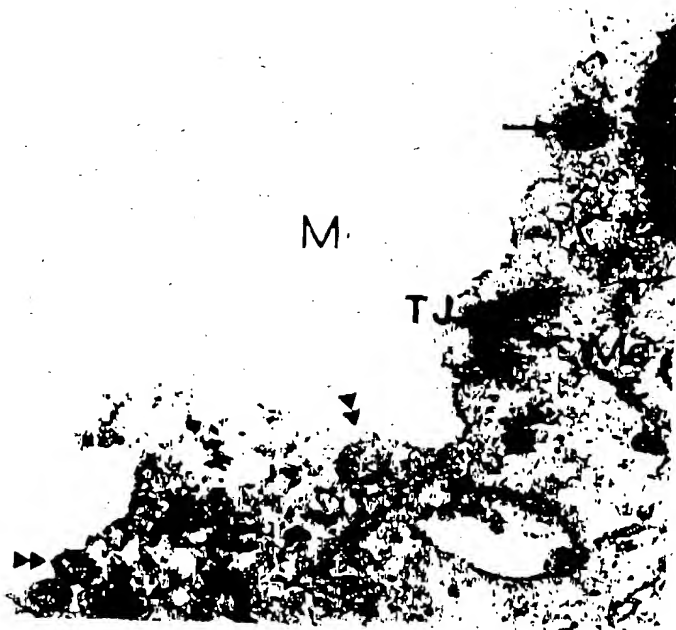


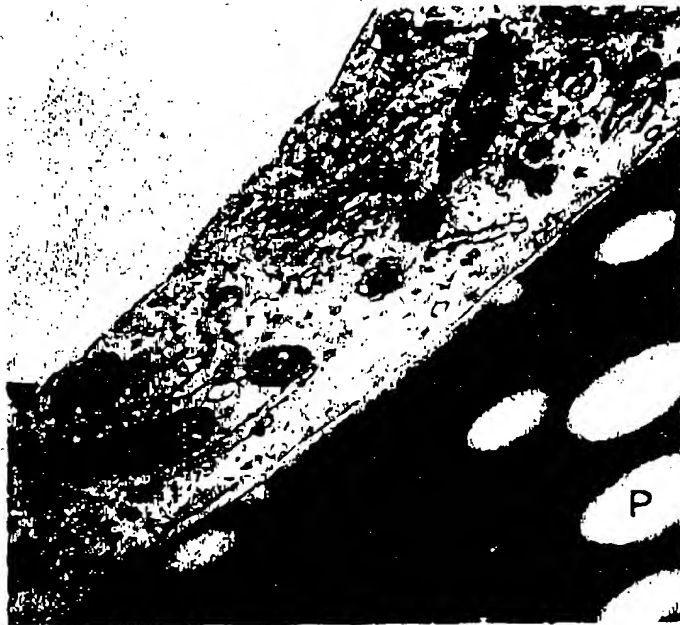
Fig. 4. Surface region of normal (*in vitro*) lymphatic endothelium showing tight junction (TJ), mitochondria (Mc), micro-projection (M) and Weibel-Palade bodies (arrow) in oblique section and (arrow heads) in cross section.  $\times 45,000$ .

interdigitating junction whereby 2 adjacent cells are joined by several finger like processes which were locked together but are still distinctly separated by their respective cellular membranes. The rarest type of cellular junction was the type of junction resembling "tight junctions". Such junctions consisted of closely opposed electron dense areas but it lacks the classical and distinctive features seen in true tight junctions in the epidermis.

Ultrastructural studies of normal (*in vivo*) lymphatic endothelium showed all the features of cultured lymphatic endothelial cells (fig. 4). On the luminal surface were varying numbers of cytoplasmic finger-like projections and also caveolae. The usual cytoplasmic organelles as well as WPB were present. Indistinct and poorly formed "tight" intercellular junctions were also observed.

#### *The ultrastructure of bovine aortic endothelial cells, human umbilical vein endothelial cells and human capillary endothelial cells*

There was generally little difference in the ultrastructural characteristics of these endothelial cells from different sources. Only minor differences exist. The main features are summarised in table 2. Cultured endothelial cells have a centrally located nucleus with one or 2 nucleoli. The cytoplasm contains the usual cytoplasmic organelles including mitochondria, rough and smooth endoplasmic reticulum, pinocytic vesicles, free and attached ribosomes, Golgi apparatus, filaments, multivesicular bodies and varying numbers of myelin bodies. On the cell surface there are blunt protrusions with varying numbers of caveolae and occasional exocytic vesicles. Weibel-Palade bodies, the unique ultrastructure of endothelial cells were



**Fig. 5.** Cultured human endothelial cell showing caveola (arrow head) and several Weibel-Palade bodies (arrows). P = gelatin substrate.  $\times 45,000$ .

present in all cultured endothelial cells examined (fig. 5). However, the number varied depending on the source of cultured cells. It was surprising to note that, contrary to popular belief, human capillary endothelial cells contained many Weibel-Palade bodies (fig. 6).

Cultured endothelial cells from the various sources maintain a close intercellular relationship. Most of the different types of cell junctions are encountered. These include simple overlapping, end to end abutment, complex interdigitating and mortice-like junctions (fig. 3). Tight junctions are frequently encountered. There are also micro-channels where pinocytic vesicles often open into (fig. 7).

#### *Immunofluorescent studies on cultured endothelial cells*

All cultured endothelial cells in this study were examined by indirect immunofluorescent microscopy for the presence of FVIII:RA. The expression of FVIII:RA has been recognised as a specific feature of endothelial cells. In this study all cultured cells were found to express FVIII:RA. All primary and subcultures of endothelial cells exhibit brilliant granular green fluorescence (fig. 8a). By using phase contrast microscopy it was possible to determine the exact proportion of cells containing FVIII:RA (fig. 8b). There was slight variation in the staining intensity but cultured cells still exhibited strong fluorescence after 8 passages. In an effort to ensure specific staining for FVIII:RA steps were taken to block non-specific staining by incubating cultured cells in diluted pig antisera or incubating cells with dilute 3% pig serum in the staining procedure. Negative controls were also included. Cultured endothelial cells were also stained with *Ulex europaeus* I lectin (UEA-I). It was found that endothelial cells showed variable staining intensity.



**Fig. 6.** Cultured human endothelial cell showing Weibel-Palade bodies.  $\times 75,000$ .

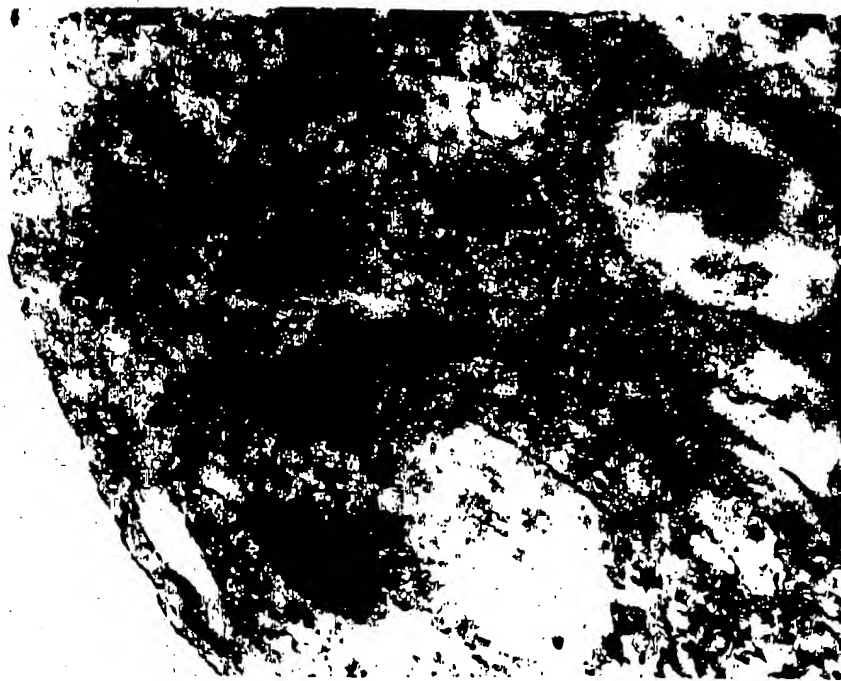


**Fig. 7.** Cultured endothelial cell showing micro-channel (arrow) and surmounting junction.

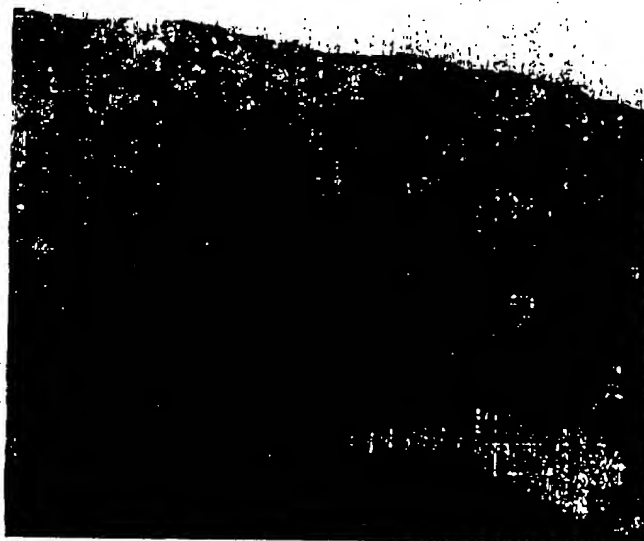
low head) and several Weibel-

However, the number varied to note that, contrary to popular belief-Palade bodies (fig. 6). in a close intercellular relationship-  
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by indirect immunofluorescent IURA has been recognised as a cell cells were found to express exhibit brilliant granular green was possible to determine the there was slight variation in the fluorescence after 8 passages. In an then to block non-specific staining cells with dilute 3% pig serum ded. Cultured endothelial cells was found that endothelial cells



**Fig. 6.** Cultured human *capillary* endothelial cell showing several Weibel-Palade bodies.  $\times 75,000$ .



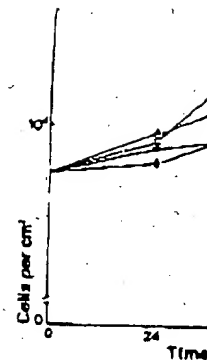
**Fig. 7.** Cultured bovine endothelium showing tight junction (TJ), an intercellular microchannel (arrow) and surface vesicle (V).  $\times 74,000$ .



**Fig. 8.** a) Cultured lymphatic endothelial cells exhibiting bright granular immunofluorescence for FVIIIIRA.  $\times 430$ . b) Phase contrast view of the same field verifying that all cells show FVIIIIRA.  $\times 370$ .

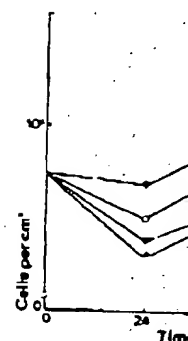
#### *Proliferation studies and doubling times*

The growth rate of various types of cultured endothelial cells were studied in different culture conditions during their logarithmic growth phase. It was established that the optimal period of determining the doubling time of cell growth was between 24 to 48 h of passage. By

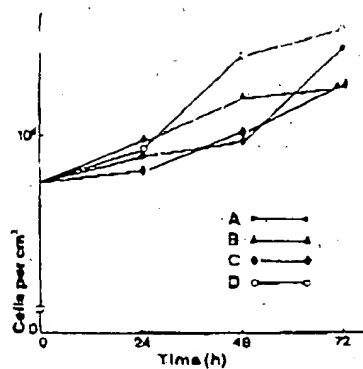


**Fig. 9.** Growth curve passage inoculated  
A = ECGF (100  $\mu$  ml), 20% FCS, at plastic; D = ECGF

**Fig. 10.** Growth curve inoculated at  $8 \times 10^4$   
A = ECGF (100  $\mu$  ml), 20% FCS and  
20% FCS and gelatin human AB negativ

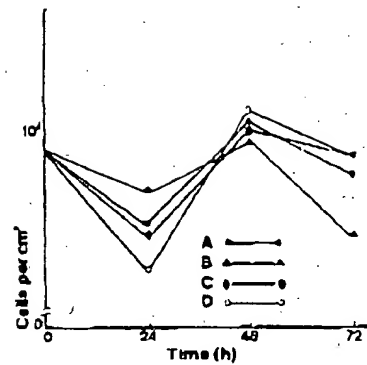


this time the cell confluence. The conditions are the same as in previous works in



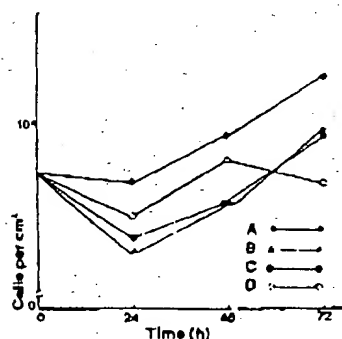
**Fig. 9.** Growth curve for cultured lymphatic endothelium after the 4th (D) and 7th (A, B, C) passage inoculated at  $5.8 \times 10^4$  cells/cm<sup>2</sup> in the following culture conditions:

A = ECGF (100 µg/ml), heparin (100 µg/ml), 20% FCS, and gelatin; B = ECGF (100 µg/ml), 20% FCS, and gelatin; C = ECGF (100 µg/ml), heparin (100 µg/ml), 20% FCS and plastic; D = ECGF (100 µg/ml), heparin (100 µg/ml), 20% FCS and gelatin.



**Fig. 10.** Growth curves for cultured human umbilical vein endothelium after 2 passages inoculated at  $8 \times 10^4$  cells/cm<sup>2</sup> in the following conditions:

A = ECGF (100 µg/ml), heparin (100 µg/ml), 20% FCS and gelatin; B = heparin (100 µg/ml), 20% FCS and gelatin; C = ECGF (crude preparation, 50 µl/ml), heparin (100 µg/ml), 20% FCS and gelatin; D = ECGF (crude preparation, 50 µl/ml), heparin (100 µg/ml), 20% human AB negative serum and gelatin.



**Fig. 11.** Growth curves for cultured human aortic endothelium after 7 passages and inoculated at  $5.4 \times 10^4$  cells/cm<sup>2</sup> in the following culture conditions:

A = ECGF (100 µg/ml), heparin (100 µg/ml), 20% human AB negative serum and gelatin; B = ECGF (100 µg/ml), 20% human AB negative serum and gelatin; C = ECGF (crude preparation, 50 µl/ml), heparin (100 µg/ml), 20% human AB negative serum and gelatin; D = heparin (100 µg/ml), 20% human AB negative serum and gelatin.

this time the cells would have recovered from the shock of passage and by 48 h reached confluence. The relative growth curves of various endothelial cells under various growth conditions are shown in figs. 9, 10 and 11. It is evident that the doubling times varied with culture conditions. Lymphatic endothelial cells grow best in culture media containing ECGF, heparin, FCS and on a substrate of gelatin. The results are compared with results from previous works in table 2.

Table 2. Growth characteristics of endothelial cells from various sources.

Type of endothelium	Culture conditions		ECGF	Heparin	Surface substrate	Doubling time	C.P.D.L.	Reference
	Culture medium	FCS						
Human umbilical vein	199	20%	—	—	plastic	92 h	ND	JAFFE et al. 1973 (5)
	199	20%	—	—	plastic	42-48 h	ND	GIBBONE 1976 (3)
	199	20%	100 µl/ml	—	fibronectin	42-72 h	27-34	MAUAG et al. 1981 (13)
	199	20%	100 µl/ml	—	gelatin	64 h	ND	THORNTON et al. 1983 (19)
	199	20%	150 µl/ml	—	fibronectin	25 h	ND	MAUAG et al. 1984 (13)
	199	20%	20 µl/ml	90 µl/ml	gelatin	18-21 h	42-79	THORNTON et al. 1983 (19)
Human aorta	199	20%	20 µl/ml	90 µl/ml	gelatin	18-21 h	42-79	THORNTON et al. 1983 (19)
Bovine aorta	RPMI-164	35%	—	—	plastic	29-34 h	ND	DOOYSE et al. 1975 (2)
	Waymouth	30%	—	—	plastic	24-48 h	35-40	SAGE 1984 (15)
Bovine lymphatic	199	20%	—	—	plastic	ND	ND	JOHNSTONE and WALKER 1984 (9)
Canine lymphatic	199	20%	—	—	plastic	36-68 h	ND	GNEPP and CHANDLER 1985 (4)
	199	20%	150 µl/ml	—	plastic	36-68 h	ND	GNEPP and CHANDLER 1985 (4)

C.P.D.L. = Cumulative population doubling level is the number of population doublings that fail to proliferate further. ND = no data available. FCS = Foetal calf serum. ECGF = Endothelial cell growth factor.

## Endothelium of normal

It is unnecessary to sufficient to say that the main purpose of including *in vitro* features of endovessels and lymphatics

## Discussion

There is considerable secretory substances with states (6). Cultured human possess intrinsic ability fibronectin, thrombospondin endothelia have a similar function as passive or system. This notion is of modulating activity information on the secret characterise and establish vascular endothelia and

This study has shown endothelium are also seen the growth characteristic growth pattern, forming stone pattern at confluence have similar ultrastructure Weibel-Palade bodies and caveolae. Some capillary endothelium that Weibel-Palade body has composition and serves as a specific marker for that cultured endothelial subcultures, but we have

The types of intercellular. However, it was noted whereas they are frequently consistent with the relationship associated with a relative

We have found that results reported by or necessary for the optimum of gelatine is the most rate and doubling time cultured endothelia. Comparison more without significance

JOHNSTONE and WALKER contain FVIII-RA. combination of immature lymphatic endothelial



### *Endothelium of normal in vivo blood vessels and lymphatic*

It is unnecessary to describe in detail the structure of normal endothelium. However, it is sufficient to say that the findings in this study are not at variance with published data. The main purpose of including normal tissues in this study is to enable a comparison of *in vivo* and *in vitro* features of endothelial cells. Results have shown that all endothelia from normal blood vessels and lymphatics contain FVIII<sup>RA</sup> and Weibel-Palade bodies.

### Discussion

There is considerable experimental evidence to show that cultured vascular endothelium secretes substances with biochemical activity important for normal function and in disease states (6). Cultured human vascular endothelia have been shown to express ABO antigens, possess intrinsic ability to form 3-dimensional networks in culture, produce prostacyclin, fibronectin, thrombospondin, and type III procollagen (6). It is not known whether lymphatic endothelia have a similar intrinsic secretory ability. It is generally assumed that lymphatics function as passive conduits returning excessive interstitial fluid to the main circulatory system. This notion is based on the current assumption that lymphatics do not have secretory or modulating activity. Past studies of the composition of lymph do not provide reliable information on the secretory activity of lymphatic endothelium. This study was designed to characterise and establish long-term cultures of lymphatic endothelia, to compare them with vascular endothelia and to subsequently investigate their pathobiological activities.

This study has shown that the current techniques for the isolation and culture of vascular endothelium are also suitable for culturing lymphatic endothelium. The culture conditions and the growth characteristics are similar for all types of endothelium. They all have a common growth pattern, forming a monolayer during the early growth period and develop a cobble stone pattern at confluence. Cultured endothelia derived from blood vessels and lymphatics have similar ultrastructural features. All cultured endothelia express FVIII<sup>RA</sup>, possess Weibel-Palade bodies and the usual cytoplasmic organelles including cell surface projections and caveolae. Some workers have questioned the existence of Weibel-Palade bodies in capillary endothelium and especially lymphatic endothelium. This study has clearly shown that Weibel-Palade bodies are present in both normal and cultured lymphatic endothelium. The Weibel-Palade body has been shown in this study to be an integral part of the cytoplasmic composition and serves as a specific ultrastructural marker for its identification just as it serves as a specific marker for the identification of vascular endothelium. This study has also shown that cultured endothelial cells have retained all the structural characteristics even after several subcultures, but we have not studied whether lymphatic endothelia are site or organ specific.

The types of intercellular cell junctions did not differ in different types of endothelia. However, it was noted that tight junctions are rarely found in cultures of lymphatic endothelia whereas they are frequently seen in cultures of endothelia from blood vessels. This is consistent with the respective functions of lymphatic and blood vessels, lymphatics being associated with a relatively low pressure system.

We have found that the growth requirements of cultured endothelia are comparable to results reported by other researchers (table 2). Growth supplements and a substrate are necessary for the optimal growth of lymphatic endothelial cells. It was found that a thin layer of gelatine is the most suitable substrate for the culture of lymphatic endothelium. The growth rate and doubling time of lymphatic endothelium were comparable with that of other types of cultured endothelia. Cultures of lymphatic endothelium can be maintained for 3 months or more without significant alteration in growth and structural characteristics.

JOHNSTONE and WALKER (9) studied cultured lymphatics and reported that not all of their cells contain FVIII<sup>RA</sup>. This is at variance with our findings. We were able to demonstrate by a combination of immunological staining and phase contrast microscopy that all our cultured lymphatic endothelial cells possess FVIII<sup>RA</sup>. The difference is probably due to a difference in



the purity of cultures or that some of JOHNSTONE and WALKER's cultured cells may have lost their expression for FVIII<sup>R</sup>A. We were also able to show that cultured lymphatic endothelial cells have Weibel-Palade bodies. JOHNSTONE and WALKER (9) did not publish any data on ultrastructural studies of their cells. Other workers have also shown variation in FVIII<sup>R</sup>A expression of lymphatic endothelia (1, 17).

Our findings that blood vascular and lymphatic endothelia have similar growth requirements, growth characteristics and have FVIII<sup>R</sup>A further support the notion of a common origin of blood and lymphatic vessels. There are 2 opposing views on the origin of lymphatic endothelium. One view is that lymphatic vessels differentiate *de novo* from local mesenchyme as a series of channels which subsequently connect centrally to form the lymphatic system. The opposing view is that the lymphatic system develops as a series of venous outpouchings during early embryonic life, the channels ramify and extend peripherally to eventually form the definitive lymphatic system (11, 20). If we accept the latter theory, it is therefore not surprising to find that endothelium from blood and lymphatic vessels share common characteristics. This does not imply that there are subtle biochemical or immunological differences between blood and lymphatic endothelium or that lymphatic endothelia may also be site and organ specific. More work, with particular emphasis on cultures of lymphatic endothelium from capillaries and a study of their antigenic expression as well as biochemical features may help to elucidate some of the issues.

The Weibel-Palade body is a unique endothelial cell structure. It has an internal substructure of parallel micro-tubules bound by a membrane. Its exact function is not fully known, but recent work has shown that it may be the site of production and/or storage of von Willebrand factor. This notion is supported by the fact that the Weibel-Palade body has been seen to be closely associated with the Golgi apparatus. Some researchers have doubted the existence of Weibel-Palade bodies in lymphatic endothelium. TABUCHI and YAMAMOTO (18) have shown Weibel-Palade bodies in canine lymphatic endothelium. There is also evidence that Weibel-Palade bodies may occur in clusters in certain types of large vessel endothelia and scarcely in endothelia of small capillaries. Our work has shown the presence of Weibel-Palade bodies in cultured and normal lymphatic endothelium. However, we have not examined the distribution or the density of Weibel-Palade bodies in lymphatic endothelium. Nevertheless, the fact that Weibel-Palade bodies are found in lymphatic endothelium and the suggestion of Weibel-Palade bodies being a source of von Willebrand factor raises the possibility that lymphatics may be an important source of von Willebrand factor in health and disease states. It is also reasonable to postulate that the structure and biochemical activity of von Willebrand factor derived from lymphatics may differ from that derived from blood vessels.

#### Acknowledgement

We gratefully acknowledge the help of Dr. R. MARKS with cultures of capillary endothelia from human foreskin.

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cultured cells may have lost  
 red lymphatic endothelial  
 and not publish any data on  
 own variation in FVIII:RA

ve similar growth require-  
 the notion of a common  
 on the origin of lymphatic  
 ex novo from local mesen-  
 ally to form the lymphatic  
 ops as a series of venous  
 and extend peripherally to  
 cept the latter theory, it is  
 d lymphatic vessels share  
 e subtle biochemical or  
 helium or that lymphatic  
 ith particular emphasis on  
 their antigenic expression  
 issues.

It has an internal substruc-  
 tion is not fully known,  
 and/or storage of von Wil-  
 bel-Palade body has been  
 earchers have doubted the  
 UCHI and YAMAMOTO (18)  
 um. There is also evidence  
 of large vessel endothelia  
 wn the presence of Weibel-  
 However, we have not  
 n lymphatic endothelium.  
 phatic endothelium and the  
 illebrand factor raises the  
 illebrand factor in health  
 structure and biochemical  
 uffer from that derived from

with cultures of capillary

in of Kaposi's sarcoma from  
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COMMONWEALTH OF AUSTRALIA

(Patents Act 1990)

IN THE MATTER OF: Australian  
Patent Application 696764  
(73941/94). In the name of:  
Human Genome Sciences Inc.

- and -

IN THE MATTER OF: Opposition  
thereto by Ludwig Institute for Cancer  
Research, under Section 59 of the  
Patents Act.

Annexure GBC-23

This is **Annexure GBC-23** referred to in my Statutory Declaration made this  
Thirteenth day of December 2000.

  
\_\_\_\_\_  
Gary Baxter Cox

WITNESS:

  
\_\_\_\_\_  
Patent Attorney

PEYTEE KIRO

Table 1 : Publications referred to in Applicant's Evidence in Answer and the relative annexure number for them.

Publication	Ludwig's Evidence in support	HGS Evidence in Answer
1. Andersson et al. (1992) <i>J. Biol. Chem.</i> 267:11260-11266.	JFM-1	
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